

**HISTONE DEACETYLASE  
INHIBITOR REGULATION OF  
GENE EXPRESSION**

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Doctor of Philosophy  
In the Department of Biochemistry  
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By  
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## ABSTRACT

Histone deacetylase inhibitors (HDIs) are a group of chemo-preventive and chemo-therapeutic agents that have generated significant attention in clinical trials, given their ability to selectively induce cell cycle arrest, differentiation and/or apoptosis of tumor cells. Presently, these agents are proposed to function by altering gene expression levels, primarily by promoting histone hyperacetylation and gene transcription. However, in this thesis, HDIs are reported to control the expression of genes from the c-Src kinase family and p21WAF1 by means other than transcriptional activation.

Overexpression and activation of c-Src, a 60kDa non-receptor tyrosine kinase, has been implicated in the development, growth, progression, and metastasis of several human cancers, especially those of the colon. Butyrate and the more specific histone deacetylase inhibitor trichostatin A (TSA) were both found to effectively inhibit the expression of c-Src mRNA and protein in a number of tumor cell lines, including those of the colon, liver and breast. Expression of the SRC oncogene is alternatively regulated by the SRC1A and SRC1 $\alpha$  promoters. HDIs were shown to repress c-Src expression by inhibiting transcription of both of these promoters, independent of any new protein synthesis. Furthermore, butyrate and TSA similarly regulated the expression of the c-Src family kinase (SFK) members Yes, Fyn, Lyn and Lck in human colon cancer cell lines. In addition, TATA binding protein (TBP) associated factor 1 (TAF1) was shown to be necessary for basal transcription of the SRC1A, YES and LYN promoters, but was not required for HDI mediated repression.

Induction of the potent cyclin dependent kinase inhibitor p21WAF1 has been identified to be a key feature of HDI mediated cell cycle arrest. The level of p21WAF1 expression has been extensively reported to be directly upregulated by HDIs in a p53 independent manner that requires Sp family binding sites in the p21WAF1 proximal promoter to induce transcription. However, HDIs were shown to be capable of inducing p21WAF1 gene expression, dependent on new protein synthesis, by increasing mRNA stability. To date, p21WAF1 mRNA stability has been extensively studied and a number of *cis*-acting elements in the 3' untranslated region (UTR) of the p21WAF1 mRNA have been implicated in the regulation of mRNA stability, such as AU rich

elements (AREs) and a 42 nucleotide HuD/Elav binding element. Similarly, in this work, two novel *cis*-acting elements were identified in the 3' UTR of p21WAF1 and were shown to facilitate basal and HDI induced post-transcriptional regulation of p21WAF1 mRNA stability in HepG2 cells. Collectively, these studies highlight the intricacy of HDI mediated effects and challenge the preconceptions regarding the molecular mechanism of these anti-tumor agents.



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## TABLE OF CONTENTS

<b>PERMISSION TO USE</b>	i
<b>ABSTRACT</b>	ii
<b>ACKNOWLEDGEMENTS</b>	iv
<b>TABLE OF CONTENTS</b>	v
<b>LIST OF TABLES</b>	xi
<b>LIST OF FIGURES</b>	xii
<b>LIST OF ABBREVIATIONS</b>	xv
<b>1. REVIEW OF THE LITERATURE</b>	1
<b>1.1. Introduction</b>	1
<b>1.2. Chromatin Structure and Remodeling</b>	2
1.2.1. Chromatin Structure	2
1.2.2. ATP-Dependent Chromatin Remodeling Enzymes	3
1.2.3. Histone Modifying Enzymes	5
1.2.3.1. Histone Acetyltransferases	6
1.2.3.2. Histone Deacetylase	8
1.2.3.3. Other Covalent Histone Modifying Enzymes	11
1.2.4. Histone Deacetylase Inhibitor Classes and Clinical Trials	12
1.2.4.1. Histone Deacetylase Inhibitor Classes and Clinical Trials	16
<b>1.3. Eukaryotic Transcriptional Regulation</b>	21
1.3.1. Promoter Architecture	21
1.3.1.1. The Core Promoter Element	23
1.3.1.2. Proximal Promoter and Enhancer Elements	24
1.3.2. Pre-initiation and RNA Polymerase II Transcriptional Machinery	25
1.3.2.1. Pre-initiation Complex Assembly and Open Complex Arrangement	25
1.3.2.2. Mediator	28
1.3.3. Transcription Initiation and Promoter Clearance	29
1.3.4. Transcript Elongation	30
1.3.4.1. From the Early Elongation Complex to Productive Elongation	30
1.3.5. Termination	31
<b>1.4. mRNA</b>	32
1.4.1. Co-transcriptional mRNA Processing Events	32

1.4.1.1. Addition of the 5' Cap Structure at the Early Elongation Pause	32
1.4.1.2. Intron Splicing During Productive Elongation	34
1.4.1.3. 3' Polyadenylation of Precursor-mRNA and Transcript Termination	35
1.4.2. <i>Cis</i> -Acting mRNA Stability Determinants	39
1.4.2.1. The mRNA Cap and 5' Untranslated Region	39
1.4.2.2. Coding Region	41
1.4.2.3. The Poly(A) Tail and 3' Untranslated Region	41
<b>1.5. c-Src and the c-Src Family Kinase Members</b>	<b>45</b>
1.5.1. Overview of the Rous Sarcoma Virus, v-Src and the c-Src Proto-oncogene	45
1.5.2. c-Src Family Kinase Protein Structure and Regulation	46
1.5.3. Signaling Events Mediated by the c-Src Family Kinase Members	49
1.5.4. c-Src Family Kinase Knockout Mice	50
1.5.5. Role of the c-Src Family Kinase Members in Cancer	51
1.5.6. SRC Gene Transcription	53
1.5.7. The c-Src Family Kinase YES, LYN and LCK Gene Promoters	56
<b>1.6. p21WAF1</b>	<b>58</b>
1.6.1. p21WAF1 Protein Function	58
1.6.2. Regulation of p21WAF1	60
1.6.2.1. p53 Dependent Regulation of p21WAF1 Transcription	61
1.6.2.2. p53 Independent Regulation of p21WAF1 Transcription	63
1.6.2.3. Histone Deacetylase Inhibitor Regulation of p21WAF1 Transcription	65
1.6.2.4. p21WAF1 mRNA stability	67
<b>2. SPECIFIC AIMS AND HYPOTHESIS</b>	<b>71</b>
<b>3. MATERIALS AND METHODS</b>	<b>73</b>
<b>3.1. Reagents, Equipment, Software and Distributors</b>	<b>73</b>
<b>3.2. Culture of cell lines</b>	<b>83</b>
3.2.1. Culture and Maintenance of Cell Lines	83
3.2.2. Histone Deacetylase Inhibitor and Cycloheximide Treatments	84
3.3.3. Actinomycin D Treatments	84
<b>3.3. Cellular Growth Curve Experiments</b>	<b>84</b>
<b>3.4. Bacterial Strains</b>	<b>85</b>
<b>3.5. General Molecular Biology Techniques</b>	<b>85</b>
3.5.1. Cloning Techniques	86

3.5.1.1. Restriction Enzyme Digestion	86
3.5.1.2. Preparation of Blunt End DNA Fragments	86
3.5.1.3. Removal of the 5' Phosphate Group of DNA	86
3.5.1.4. Polymerase Chain Reaction	86
3.5.1.5. Annealing of Complementary Oligonucleotides	87
3.5.1.6. Agarose Gel Electrophoresis	87
3.5.1.7. Gel Extraction of Linear DNA Fragments	87
3.5.1.8. DNA Ligation Reaction	88
3.5.2. QuickChange Site-Directed Mutagenesis	88
3.5.3. Competent Bacterial Cells	88
3.5.3.1. Preparation of TOP10 cells	88
3.5.3.2. Transformation of Competent TOP10 Cells	89
3.5.4. Zero Blunt TOPO PCR Cloning and One Shot TOP10 Cell Transformation	89
3.5.5. Sequencing	90
3.5.6. Plasmid Isolation from <i>E. coli</i> Strains	90
3.5.6.1. Small-Scale Plasmid Isolation	90
3.5.6.2. Mid-Scale Endofree Plasmid Isolation	90
3.5.6.3. Endofree Isolation of Large Scale Plasmid Preparations	91
3.5.7. DNA Ladder Markers	91
<b>3.6. Information on Plasmids and Construction Details</b>	91
3.6.1. Expression Vectors and cDNAs	91
3.6.2. c-Src Family Kinase CAT Reporter Constructs	92
3.6.3. p21WAF1 Reporter Constructs	92
3.6.4. -210WAF1-CAT + p21WAF1 3'UTR Reporter Vectors	93
3.6.4.1. -210WAF1-CAT + p21WAF1 3'UTR 5' Deletion Plasmids	93
3.6.4.2. -210WAF1-CAT + p21WAF1 3'UTR Internal Deletion Plasmids	94
3.6.4.3. -210WAF1-CAT + p21WAF1 3'UTR 3' Deletion Plasmids	94
3.6.4.4. -210WAF1-CAT + p21WAF1 3'UTR Mutant Constructs	95
3.6.4.5. -210WAF1-CAT + p21WAF1 3'UTR + Poly(A) Signal Constructs	95
<b>3.7. Transient Transfections of Cultured Cells</b>	96
3.7.1. Standard Transfection Conditions	96
3.7.2. Histone Deacetylase Inhibitor Transfection Procedure	97
3.7.3. Transfection of BHK-21 and <i>tsBN462</i> Cells	97
3.7.3.1. BHK-21 and <i>tsBN462</i> Transfections and Histone Deacetylase Inhibitors	97
3.7.3.2. <i>tsBN462</i> Co-transfections	98

<b>3.8. Determination of CAT Reporter Activity</b>	98
3.8.1. Chloramphenicol AcetylTransferase Assay	98
3.8.2. Beta-Galactosidase Assay	99
3.8.3. Bradford Protein Assay	99
<b>3.9. RNA Isolation from Cultured Cells</b>	100
<b>3.10. Northern Blot</b>	100
3.10.1. Northern Blot Electrophoresis and Gel Transfer	100
3.10.2. Northern Probes	101
3.10.3. Northern Blot Hybridization	102
<b>3.11. Western Blot</b>	102
3.11.1. Lowry Protein Assay	102
3.11.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	102
3.11.3. Western Blot Method and Antibodies	103
<b>3.12. Isolation of Nuclear and Cytoplasmic Cellular Extracts</b>	103
<b>3.13. RNA Mobility Shift Assays (RMSAs)</b>	104
3.13.1. Preparation of the T7 Transcript	104
3.13.2. T7 Reaction and RNA probe isolation	104
3.13.3. RNA Mobility Shift Assay Method	105
<b>4. RESULTS</b>	106
<b>4.1 THE EFFECT OF HISTONE DEACETYLASE INHIBITORS ON THE GENE EXPRESSION LEVELS OF C-SRC</b>	106
4.1.1. The Influence of Histone Deacetylase Inhibitors on Tumor Cell Growth	106
4.1.2. Histone Deacetylase Inhibitor Downregulation of c-Src Expression Levels	107
4.1.3. The Effect of Histone Deacetylase Inhibitors on SRC Transcription	113
4.1.4. p21WAF1 Activation of SRC Transcription	115
4.1.5. Downregulation of c-Src Expression Levels by Histone Deacetylase Inhibitors does not Require New Protein Synthesis	115
4.1.6. SUMMARY	118
<b>4.2. THE RESPONSE OF C-SRC FAMILY KINASE MEMBERS TO HISTONE DEACETYLASE INHIBITORS</b>	119
4.2.1. The Relative Expression Patterns of c-Src Family Kinase Members in Human Colon Cancer Cell Lines	119
4.2.2. Histone Deacetylase Inhibitors Decrease c-Src Family Kinase Expression Levels	121
4.2.3. The Effect of Cycloheximide on Histone Deacetylase Inhibitor Mediated Repression of the c-Src Family Kinase Members	123

4.2.4. Promoters of c-Src Family Kinase Members are Downregulated by Histone Deacetylase Inhibitors	127
4.2.5. TAF1 Dependency of the c-Src Family Kinase Member Promoters	129
4.2.6. Activity of the TAF1 Acetyltransferase Domain is Required for c-Src Family Kinase Member Transcription	130
4.2.7. SUMMARY	135
<b>4.3. HISTONE DEACETYLASE INHIBITOR REGULATION OF P21WAF1 IN HEPG2 CELLS</b>	136
4.3.1. New Protein Synthesis is Required for HDI Regulation of p21WAF1 in HepG2 cells	136
4.3.2. Histone Deacetylase Inhibitors Cannot Activate the p21WAF1 Promoter in HepG2 Cells	136
4.3.3. p21WAF1 mRNA is Regulated Post-transcriptionally by Histone Deacetylase Inhibitors	138
4.3.4. The Role of the p21WAF1 3' Untranslated Region in Histone Deacetylase Inhibitor Mediated Stabilization of p21WAF1 mRNA	140
4.3.4.1. Proximal p21WAF1 3' Untranslated Region Deletions	145
4.3.4.2. Further 5' Deletions of the p21WAF1 3' Untranslated Region	148
4.3.4.3. Deletions of the p21WAF1 3' Untranslated Region at the Extreme 3' end	151
4.3.4.4. p21WAF1 3' Untranslated Region Mutants	151
4.3.5. SUMMARY	163
<b>5. GENERAL DISCUSSION</b>	165
<b>5.1. Histone Deacetylase Inhibitors Function as Anti-neoplastic Agents</b>	165
<b>5.2. The Mechanisms of Histone Deacetylase Inhibitor Action</b>	166
<b>5.3. Histone Deacetylase Inhibitor Repression of c-Src and the c-Src Family Kinase Members</b>	168
5.3.1. c-Src Gene Expression is Suppressed by HDIs	168
5.3.2. c-Src Family Kinase Member Expression is Downregulated by Histone Deacetylase Inhibitors	168
5.3.3. The Mechanism of Histone Deacetylase Inhibitor Directed Inhibition of c-Src and the c-Src Family Kinase Members	169
5.3.3.1. The Involvement of TAF1	170
5.3.3.2. Other Potential Mechanisms of Histone Deacetylase Inhibitor Mediated SRC Repression	174
5.3.3.3. The Working Model of Histone Deacetylase Inhibitor Mediated Downregulation of c-Src Gene Expression	177
5.3.4. The Importance of c-Src Family Kinase Member Gene Repression by Histone Deacetylase Inhibitors	180

<b>5.4. Post-transcriptional Regulation of p21WAF1 by Histone Deacetylase Inhibitors</b>	181
5.4.1. The Mechanism of Histone Deacetylase Inhibitor Mediated p21WAF1 mRNA Stabilization	182
5.4.1.1. Histone Deacetylase Inhibitor Mediated Stabilization of p21WAF1 mRNA by Two <i>cis</i> -acting Sequences in the p21WAF1 3' Untranslated Region	183
5.4.2. The Model of Histone Deacetylase Inhibitor Regulation of p21WAF1 mRNA Stability	186
<b>5.5. Scope and Significance</b>	188
<b>6. CONCLUSIONS AND FUTURE STUDIES</b>	189
<b>7. REFERENCES</b>	194

## LIST OF TABLES

Table 1.1. Histone deacetylase inhibitors, classes, structures, and status of clinical trials	17
Table 1.2. mRNA <i>cis</i> -acting stability determinants of the 5' UTR and coding region	40
Table 1.3. <i>Cis</i> -acting stability determinants of the mRNA 3' UTR	43
Table 3.1. The List of Reagents and Distributors	73
Table 3.2. Commercial Kits and Distributors	76
Table 3.3. Equipment & Software and Distributors	77
Table 3.4. Distributor Addresses	77
Table 3.5. PCR Primers and Oligonucleotides	79
Table 4.1. A summary table comparing relative basal CAT activities and CAT fold induction following HDI exposure in HepG2 transfected cells with the -210WAF1-CAT + p21WAF1 3'UTR deletion and mutation plasmids	164



## LIST OF FIGURES

Figure 1.1. Eukaryotic promoter architecture and components of the RNA Polymerase II transcriptional machinery	22
Figure 1.2. The protein structure of TAF1	27
Figure 1.3. Polyadenylation of the pre-mRNA	36
Figure 1.4. The protein structure of chicken c-Src	48
Figure 1.5. The human SRC gene	54
Figure 1.6. Gene promoters of human c-Src family kinase members	57
Figure 1.7. Regulation of the p21WAF1 promoter	64
Figure 1.8. The p21WAF1 mRNA and the <i>trans</i> -acting RNA binding proteins that regulate p21WAF1 mRNA stability in response to various agents	69
Figure 4.1. Sodium butyrate decreases cell growth in human colon and breast tumor cell lines	108
Figure 4.2. Histone deacetylase inhibitors decrease cell growth in HepG2 cells	109
Figure 4.3. Sodium butyrate suppresses c-Src mRNA levels dose dependently in human tumor cells	111
Figure 4.4. c-Src mRNA and protein levels are repressed by histone deacetylase inhibitors in human tumor cells	112
Figure 4.5. Sodium butyrate mediated repression of the SRC1 $\alpha$ and SRC1A promoters	114
Figure 4.6. The SRC1 $\alpha$ and SRC1A promoters are trans-activated by p21WAF1 in HepG2 cells	116
Figure 4.7. c-Src mRNA levels are reduced by sodium butyrate independent of new protein synthesis	117
Figure 4.8. Differential gene expression of the c-Src family kinase members in human colon cancer cell lines	120
Figure 4.9. Sodium butyrate inhibits mRNA expression of the c-Src family kinase members in human colon cancer cell lines	122
Figure 4.10. Trichostatin A represses the mRNA expression levels of the SFK members in HT29 and Colo 320 cells	124

Figure 4.11. TSA inhibits the mRNA expression levels of SFK members in SW480 and Colo 201 cells	125
Figure 4.12. Sodium butyrate represses Lck and Lyn mRNA expression levels independent of protein neo-synthesis	126
Figure 4.13. Histone deacetylase inhibitors downregulate the activity of the SRC1A, LCK type I, LYN and YES promoters	128
Figure 4.14. c-Src family kinase member promoters are TAF1 dependent	131
Figure 4.15. Structural domains of TAF1	133
Figure 4.16. TAF1 acetyltransferase activity is required to restore SFK promoter activity	134
Figure 4.17. Histone deacetylase inhibitor upregulation of p21WAF1 gene expression in HepG2 cells requires protein neo-synthesis	137
Figure 4.18. The WAF1 promoter is not activated by histone deacetylase inhibitors in HepG2 cells	139
Figure 4.19. Histone deacetylase inhibitors increase the stability of p21WAF1 mRNA in HepG2 cells	141
Figure 4.20. The sequence of the p21WAF1 3' untranslated region	142
Figure 4.21. The p21WAF1 3' UTR plays a role in histone deacetylase inhibitor mediated stabilization in HepG2 cells	143
Figure 4.22. The -210WAF1-CAT + p21WAF1 3'UTR proximal deletion constructs	146
Figure 4.23. The p21WAF1 3' UTR, HuD and AU-rich elements do not play a role in HDI mediated p21WAF1 mRNA stabilization	147
Figure 4.24. Further -210WAF1-CAT + p21WAF1 3'UTR 5' deletions	149
Figure 4.25. 5' Deletions of the p21WAF1 3' UTR highlight a potential <i>cis</i> -acting sequence	150
Figure 4.26. The -210WAF1-CAT + p21WAF1 3'UTR extreme 3' deletions	152
Figure 4.27. 3' Deletions of the p21WAF1 3' UTR suggest the presence of a second <i>cis</i> -acting element	153
Figure 4.28. High sequence conservation exists within the extreme 3' region of the p21WAF1 3' UTR	155

Figure 4.29. p21WAF1 3' UTR mutants #1 through #8 are still incapable of enhancing mRNA stability	156
Figure 4.30. The p21WAF1 3' UTR mutants #15 and #16 have compromised mRNA stabilization ability in the presence of histone deacetylase inhibitors	157
Figure 4.31. A diagram of the -210WAF1-CAT + p21WAF1 3'UTR wt + poly(A) hexamer vector	159
Figure 4.32. Nucleotides 2071-2079 of the p21WAF1 3' UTR contain an instability determinant and a histone deacetylase inhibitor responsive element	160
Figure 4.33. A change in <i>trans</i> -binding factors to the 3' UTR of the p21WAF1 mRNA does not occur following histone deacetylase inhibitor treatment	162
Figure 5.1. The model of histone deacetylase inhibitor mediated SRC gene repression	178
Figure 5.2. The model of histone deacetylase inhibitor induced p21WAF1 mRNA stabilization	187

## LIST OF ABBREVIATIONS

Act. D	Actinomycin D
AML	acute myelogenous leukemia
APL	acute promyelocytic leukemia
AR	androgen receptor
ARE	AU-rich element
AT	acetyltransferase
ATP	adenosine triphosphate
ATRA	all-trans-retinoic acid
AUF1	AU-rich binding factor 1 or hnRNP D
Bax	Bcl2-associated X protein
BCL6	B-cell lymphoma 6
BHK-21	baby hamster kidney cell line
BRCA1	Breast cancer gene 1
BRE	TFIIB-recognition element
BRG1	Brahma-related gene 1
C	carboxy
CAP	chromatin associated protein
CARM1	co-activator-associated R-methyltransferase
CAT	Chloramphenicol acetyltransferase
CBC	cap binding complex
CBP	CREB binding protein
Cdk	cyclin dependent kinase
CEs	capping enzymes
C/EBP $\alpha$	CCAAT/enhancer binding protein- $\alpha$
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CIP1	Cdk-interacting protein 1
CKI	cyclin dependent kinase inhibitor
CK2	casein kinase 2
Co-REST	co-repressor to RE1 silencing transcription factor

CRD	coding region determinant
CREB	cyclic AMP response element binding protein
CRSP	cofactor required for Sp1 activation
CSF1R	colony stimulating growth factor 1 receptor
CTCL	cutaneous T-cell lymphoma
CTD	carboxy-terminal domain
DCE	downstream core element
DMEM	Dulbecco's Modified Eagle's Medium
DMT1	DNA methyltransferase 1
DMT-3B	DNA methyltransferase-3B
Dot1	disruptor of telomeric silencing 1
DPE	downstream promoter element
DRB	5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole
DRIP	vitamin D receptor-interacting proteins
DSIF	DRB sensitivity inducing factor
E	glutamate
EBE	Ets binding protein
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELAV	embryonic lethal abnormal visual
ELL	eleven-nineteen lysine-rich in leukemia
EST	Expressed Sequence Tag
FACT	facilitates chromatin transcription
FAK	focal adhesion kinase
FAP	Familial adenomatous polyposis
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FK228	depsipeptide
GASC1	gene amplified in squamous cell carcinoma 1
GATA-1	GATA binding protein 1 (globin transcription factor 1)

Gcn5	general control nonderepressible-5
GPCR	G-protein coupled receptor
GM-CSF	granulocyte macrophage-colony stimulating factor
GNAT	Gcn5-related N-acetyltransferase
GTF	general transcription factor
HAT	histone acetyltransferase
HBO1	histone acetyltransferase bound to origin recognition complex
HCCL	human colon cancer cell line
HDAC	histone deacetylase
Hda1	histone deacetylase 1
HDI	histone deacetylase inhibitor
HMG	high mobility group protein
HMT	histone methyltransferase
HNF-1	hepatocyte nuclear factor-1
hnRNP	heterogeneous nuclear ribonuclear protein
HP1	heterochromatin-associated protein 1
Hsp90	heat shock protein 90
hACF	human ATP-utilizing chromatin assembly and remodeling factor
hBRM	human Brahma
hCHRAC	human chromatin-accessibility factor
I	isoleucine
IGF1R	insulin growth factor receptor 1 receptor
IGF I	insulin-like growth factor I
IGF II	insulin-like growth factor II
INF- $\gamma$	interferon gamma
Inr	Initiator
IRE	iron responsive element
IRP	iron regulatory protein
ISWI	imitation switch
JHDM3A	jumonji C-domain-containing histone demethylase 3A
K	lysine

KMT	lysine methyltransferase
LB	Luria-Bertani
LSD1	lysine specific demethylase 1
M	methionine
mCRD	major coding region determinant
MDM2	mouse double minutes 2
MEF2	myocyte enhancer factor 2
MeCP2	Methyl-CpG binding protein 2
miRNA	microRNA
MORF	MOZ-related factor
MOZ	monocytic leukemia zinc finger protein
MTE	motif ten element
NaB	sodium butyrate
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
N-CoR	nuclear receptor co-repressor
NELF	negative elongation factor
NES	nuclear export signal
NLS	nuclear localization signal
NuRD	nucleosome remodeling histone deacetylase
OA	okadaic acid
ORC	origin recognition complex
OTF	octamer transcription factor
P	proline
p80/XPD	gene defective in xeroderma pigmentosum patients complementation group D
p89/XPB	gene defective in xeroderma pigmentosum patients complementation group B
p21WAF1	21 kDa protein wild-type p53 activated fragment 1
PABP	poly(A) binding protein (cytoplasmic)
PABP II	poly(A) binding protein II (nuclear)
PADI4	peptidyl arginine deiminase 4

PBS	phosphate-buffered saline
PCAF	p300 CREB-binding protein- associated factor
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PGC	polycomb group protein complex
PI 3-kinase	phosphatidylinositol 3-kinase
PI3KK	phosphatidylinositol 3-kinase-like kinase
PIC	pre-initiation complex
PKC	protein kinase C
PLZF	promyelocytic leukemia zinc finger
PMA	phorbol myristate acid
PML	promyelocytic leukaemia
PP1	protein phosphatase 1
PRMT	protein arginines methyltransferase
PTCL	peripheral T-cell lymphoma
P-TEFb	Positive transcription elongation factor b
R	arginine
RAR	retinoic acid receptor- $\alpha$
RE	responsive element
RIZ	retinoblastoma-interacting zinc finger
RMSA	RNA mobility shift assay
RNA pol II	RNA polymerase II
Rpd3	reduced potassium dependency 3
RTK	receptor protein tyrosine kinase
S	serine
SAHA	suberoylanilide hydroxamic acid
SAM	S-adenosylmethionine
SANT	SWI/SNF, ADA, N-CoR and TFIIIB
Sas2	something about silencing 2
Sas3	something about silencing 3



sdi1	senescent cell-derived inhibitor 1
SET	SUV39, Enhancer of zeste and trithorax
SFK	c-Src family kinase
Sin3	switch independent 3
Sir2	silent information regulator 2
SLIDE	SANT-like ISWI domain
Smad	Sma and Mad-related protein
SMRT	silencing mediator for retinoic acid and thyroid hormone receptor
SNF2h	sucrose non fermenting 2 homologue
snRNP	small nuclear ribonucleoprotein
Sp1	specificity factor 1
SPT	suppressor that offset transcription
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-related modifier
SUV39	suppression of position-effect variegation 39
SWI2	switching 2
SWI/SNF	switching/sucrose non-fermenting
T	threonine
TAF	TBP- associated factor
TBP	TATA binding protein
TFII	transcription factor for RNA polymerase II
TGF- $\beta$	transforming growth factor beta
Tip60	Tat-interactive protein 60 kDa
TNF- $\alpha$	tumor necrosis factor alpha
TRAP/SMCC	thyroid hormone receptor-associated proteins/SRB (suppressors of RNA pol B) - Med containing cofactor
ts	temperature sensitive
TSA	trichostatin A
TTP	tristetraprolin
U2AF	U2 snRNP auxillary factor
UNR	upstream of N-Ras

UTR	untranslated region
UVC	ultraviolet light
VEGF	vascular endothelial growth factor
v-Src	viral-Src
XCPE1	X core promoter element 1
Y	tyrosine
YY1	yin yang 1

## **1. REVIEW OF THE LITERATURE**

### **1.1. Introduction**

Histone deacetylase inhibitors (HDIs) are exciting chemotherapeutic agents that have recently gained acceptance for clinical use, due to their ability to promote cell cycle arrest, differentiation and/or apoptosis. However, the mechanisms by which HDIs exhibit these effects are poorly understood. A large fraction of the literature has focused on the model that HDIs inhibit histone deacetylase enzymes and therefore shift the steady state balance toward elevated histone acetylation levels. This is proposed to relieve the compacted state of the DNA backbone, thereby enhancing transcription factor accessibility and promoting gene transcription. However, HDIs also repress a significant percentage of genes, suggesting that additional mechanisms must be responsible for this phenomena. There is increasing evidence that HDIs do, in fact, display supplementary functions beyond influencing histone acetylation and upregulating gene expression. Novel extensions of HDI regulatory events concerning the c-Src proto-oncogene and cyclin dependent kinase inhibitor p21WAF1 are presented in this thesis. c-Src is overexpressed and/or activated in a number of human malignancies and downregulation of c-Src expression levels have been hypothesized to decrease tumor cell growth. p21WAF1 is upregulated in a p53 dependent and p53 independent manner to induce cell cycle arrest. The effects that HDIs impose on gene expression of the c-Src and p21WAF1 genes have been investigated at the transcriptional and post-transcriptional levels, respectively. The literature review will provide current knowledge related to chromatin structure, histone modifying enzymes and histone deacetylase inhibitors. In addition, a review of transcriptional and post-transcriptional regulation will be touched upon. Finally, background will be presented on c-Src and p21WAF1.

## **1.2. Chromatin Structure and Remodeling**

### **1.2.1. Chromatin Structure**

Long double helix DNA molecules are efficiently packaged within a eukaryotic nucleus into distinct chromosome structures by increasing levels of DNA condensation. The nucleosome core particle is the primary unit of chromatin structural organization, consisting of 147 base pairs of DNA wrapped in 1.67 left-handed superhelical turns around a core histone octamer. The histone octamer is made up of two copies of each histone protein; H2A, H2B, H3 and H4 (Woodcock and Dimitrov, 2001 and Richmond and Davey, 2003). Two pairs of each of the histone dimers, H3-H4 and H2A-H2B, interact to form the histone core octamer (Eickbush and Moudrianakis, 1978 and Benbow, 1992). The octamer is assembled first by the association of two H3-H4 dimers, forming a H3-H4 tetramer bound to the center of the DNA, and secondly by the interaction of each H2A-H2B dimer with the tetramer, via associations between H2B and H4 (Luger *et al.*, 1997). Each of these core histone proteins contains a structured globular domain that mediates histone - histone and histone - DNA interactions, and an unstructured amino-terminal domain or tail which emanates from the surface and serves as a substrate for histone modifying enzymes to introduce post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitylation and sumoylation (Luger, 2006 and Cosgrove and Wolberger, 2005).

Chromatin is compacted into a higher order structure, known as the secondary or 30 nm structure, by repetitive folding of adjacent nucleosomes (Benbow, 1992). Condensation is primarily mediated by the linker histone, H1, which resides in a 1:1 ratio with the histone core octamer and associates within 10-90 base pairs of linker DNA beyond the core nucleosome particle (Woodcock and Dimitrov, 2001; Adkins *et al.*, 2004 and Richmond and Davey, 2003). As well, an unmodified region of the H4 N-terminal tail, between amino acids 14-19, plays a pivotal role in assembling the 30 nm chromatin fiber, likely via its interaction with the H2A acidic patch of adjacent nucleosomes (Dorigo *et al.*, 2003 and Luger and Hansen, 2005). More specifically, H4 lysine (K) residue 16 has recently been implicated in chromatin compaction, as

acetylation of H4 K16 inhibits assembly of the 30 nm chromatin fiber (Shogen-Knaak *et al.*, 2006 and Robinson and Rhodes, 2006).

Further chromatin condensation into thick fibrous mitotic chromosomes or tertiary chromatin structures occurs by poorly defined long-range chromatin fiber interactions with chromatin associated proteins bound to nucleosomes (Woodcock and Dimitrov, 2001; Adkins *et al.*, 2004; Luger and Hansen, 2005 and McBryant *et al.*, 2006). An example of this includes the association of human Methyl-CpG binding protein 2 (MeCP2) with unmethylated nucleosomal arrays, which guides chromatin condensation and generates a novel tertiary chromatin structure observed as oligomers of ellipsoidal particles (Georgel *et al.*, 2003 and McBryant *et al.*, 2006). *Drosophila* heterochromatin-associated protein 1 (HP1) and the polycomb group protein complex (PGC) represent additional examples of chromatin associated proteins involved in tertiary chromatin structure formation (Woodcock, 2006 and McBryant *et al.*, 2006).

While three distinct levels of chromatin structure organization have been outlined above, in reality the scale of chromatin condensation is rather dynamic and structurally heterogeneous, allowing numerous nuclear events such as transcription, repair, replication and recombination to occur (Luger, 2006). Even so, two principal forms of chromatin, heterochromatin and euchromatin, have been denoted at opposite ends of the spectrum. Heterochromatin is representative of a compact or inaccessible chromatin structure inherent to transcriptionally inactive regions of DNA and is present at the chromosome centromere and telomeric regions (Cosgrove and Wolberger, 2005). Euchromatin, on the other hand, is typical of uncondensed chromatin lacking histone H1, is amenable to gene activation and exists in gene rich environments (Benbow, 1992). ATP-dependent chromatin remodeling complexes and histone modifying enzymes are two of the factors known to modulate the structure of chromatin, which will be discussed in further detail.

### **1.2.2. ATP-Dependent Chromatin Remodeling Factors**

Chromatin remodeling enzymes are part of large multi-subunit protein complexes that utilize the energy derived from ATP hydrolysis to mobilize nucleosomes, thereby altering the accessibility of binding proteins to DNA target sites

(Cosgrove *et al.*, 2004). As a result, these factors play a role in regulating transcription. The best characterized ATPase subunits of these chromatin remodeling complexes belong to the Switch 2 (SWI2) and Imitation switch (ISWI) families (Eberharter and Becker, 2004). The SWI2 is the remodeling ATPase subunit of the yeast Switch/Sucrose non-fermenting (SWI/SNF) complex, and can be structurally described by a conserved ATPase and C-terminal bromodomain capable of binding acetylated lysine residues (Eberharter and Becker, 2004 and Saha *et al.*, 2006). Human homologues of the SWI2 family include Brahma-related gene 1 (BRG1) and the human Brahma (hBRM) ATPase subunits (Lusser and Kadonaga, 2003). The ISWI-based chromatin remodeling complexes include the human chromatin-accessibility factor (hCHRAC) and human ATP-utilizing chromatin assembly and remodeling factor (hACF) complexes, which both contain the ISWI ATPase subunit Sucrose non fermenting 2 homologue (SNF2h) (Corona and Tamkun, 2004). Furthermore, the ISWI family is characterized by an ATPase domain and two additional domains termed SANT and SLIDE that may be important for nucleosome and DNA binding, respectively (Corona and Tamkun, 2004 and Saha *et al.*, 2006). Interestingly, SWI2 and ISWI-containing complexes appear to regulate transcription in different ways. The SWI2-supported chromatin remodeling complexes generally disorder and reorganize nucleosomes to generate a transcriptionally accessible and competent DNA template. In contrast, ISWI-based complexes typically organize nucleosomes into precisely spaced, ordered arrays to promote transcriptional repression (Johnson *et al.*, 2005 and Saha *et al.*, 2006).

The mechanisms responsible for ATP-dependent chromatin remodeling factors disrupting and re-establishing the interactions between the core histone and DNA backbone to facilitate nucleosome rearrangement or nucleosome ‘sliding’ are not well understood. However, numerous theories have been proposed including those of the ‘twist diffusion’ and ‘bulge diffusion’ models (Flaus and Owen-Hughes, 2004 and Langst and Becker, 2004). The ‘twist diffusion’ model hypothesizes that DNA twisted at the edge nucleosome is transmitted around the histone octamer, ultimately creating new histone - DNA contacts and changing the position of the nucleosome (Langst and Becker, 2004). The ‘bulge diffusion’ model proposes that DNA - histone contacts are

broken at the edge of the nucleosome and new contacts are made with outlying DNA sequences, creating a DNA bulge that is propagated around the nucleosome, thus causing nucleosome movement (Flaus and Owen-Hughes, 2004). Of further interest, in *Saccharomyces cerevisiae*, chromatin remodeling complexes have also been documented to catalyze histone H2A-H2B dimer removal or the exchange of H2A-H2B canonical histone dimers for histone H2A.Z-H2B variants, in an ATP-dependent manner (Bruno *et al.*, 2003; Flaus and Owen-Hughes, 2004; Kobor *et al.*, 2004; Mizuguchi *et al.*, 2004 and Varga-Weisz and Becker, 2006). Therefore, ATP-dependent chromatin remodeling complexes may not only moderate chromatin structure by causing the effect of nucleosome relocation, but may also influence the composition of nucleosome histones during gene regulation (Jin *et al.*, 2005).

### **1.2.3. Histone Modifying Enzymes**

Histone modifications, in addition to DNA methylation contributes to “epigenetic regulation”. This refers to a heritable range of gene regulation controlled by modifications not inherently encoded in the genetic information derived from DNA (Santos-Rosa and Caldas, 2005; Lin *et al.*, 2006 and Shilatifard, 2006). Histone modifiers encompass a broad category of enzymes responsible for introducing post-translational covalent modifications onto histone proteins. Currently, about 150 different modifications have been identified on specific histone residues with the majority of modifications instituted on the amino terminal tails, while fewer are located on the histone globular domains (Cosgrove and Wolberger, 2005). Histone modifications include: acetylation of lysine residues, methylation of lysines and arginines (R), phosphorylation of serine (S) and threonine (T) residues, ubiquitylation as well as sumoylation of lysines (Zhang and Dent, 2005 and Luger, 2006). Histone modifying enzymes that catalyze the accumulation of chromatin post-translational modifications on the amino-terminal tails of histones have been theorized to lay down a code, referred to as the “histone code”, which may be deciphered by chromatin-associated proteins that recognize the pattern of modifications, and ultimately carry out changes in chromatin organization and gene regulation (Jenuwein and Allis, 2001 and Kouzarides, 2002). Therefore, histone modifications modulate chromatin organization

and regulate many nuclear processes, including transcription (Zhang and Dent, 2005 and Santos-Rosa and Caldas, 2005). In the following section different histone modifiers will be discussed, with particular emphasis on this histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes.

#### **1.2.3.1. Histone Acetyltransferases**

Histone acetyltransferase (HAT) enzyme activity has long been hypothesized to disrupt the electrostatic interactions between the negatively charged phosphodiester DNA backbone and the positively charged amino-terminal histone tails; when an acetyl group from acetyl coenzyme A is transferred to the epsilon nitrogen atom of the lysine residues the positive charge is neutralized (Allfrey *et al.*, 1964). The theory follows that consequent weakening of histone - DNA electrostatic interactions leads to a more relaxed chromatin structure that is open to transcription factor target site binding. In addition, as observed upon histone H4 K16 acetylation, histone acetylation inhibits nucleosome – nucleosome interactions, thereby strengthening the role of HATs as transcriptional activators (Shogen-Knaak *et al.*, 2006). HATs also have a supplementary function in transcription, as acetylated histone lysine residues may be involved in the recruitment of bromodomain-containing co-activator proteins (Zeng and Zhou, 2002). Furthermore, several HATs have been identified as components of large functional multi-protein complexes that can associate with combinations of transcriptional adaptor proteins, TATA binding protein (TBP)-associated factors (TAFs) and other HATs. Such associations further illuminate the role of HATs as transcriptional co-activators (Berger, 1999).

HATs can be divided into two separate classes that differ in origin and function. While A-type HATs are nuclear in origin and are involved in generating chromatin-based histone modifications, the B-type HATs are cytoplasmic and responsible for chromatin assembly (Roth *et al.*, 2001). To date several A-type HATs have been well characterized, including those from the Gcn5-related N-acetyltransferase (GNAT) and MYST families. p55 was the first A-type HAT discovered in *Tetrahymena thermophila* (Brownell *et al.*, 1996). Due to high sequence similarity, this led to the identification of a previously classified yeast transcriptional co-activator, general control nonderepressible-5 (Gcn5), as a HAT (Brownell *et al.*, 1996 and Kuo and Allis, 1998).



Gcn5 serves as the archetype for the GNAT family, which has been expanded to include two human HATs: hGCN5 and p300 CREB-binding protein-associated factor (PCAF). These proteins are categorized by a carboxy terminal bromodomain, a HAT domain, and an N-terminal region responsible for nucleosome recognition (Sternier and Berger, 2000). Another family of HATs, known as the MYST family, is named after its members: MOZ (monocytic leukemia zinc finger protein), Ybf2/Sas3 (something about silencing 3, also named Ybf2), Sas2 (something about silencing 2) and Tip60 (Tat-interactive protein 60kDa). Tip60, MOZ, MOZ-related factor (MORF) and histone acetyltransferase bound to origin recognition complex (HBO1) are four members of the MYST family that display HAT activity in humans (Sternier and Berger, 2000, Champagne *et al.*, 2001 and Roth *et al.*, 2001). In addition to the GNAT and MYST family members, other proteins have also been found to possess HAT activity in humans, including p300, its highly related homologue CREB binding protein (CBP), basal transcription factor TAF1, and several nuclear receptor co-activators (Kuo and Allis, 1998 and Sternier and Berger, 2000). Of particular note among these nuclear co-activators is steroid receptor co-activator-1, activator of retinoid receptors and transcriptional intermediary factor 2 (Kuo and Allis, 1998 and Sternier and Berger, 2000).

Interestingly, while histones were the first elucidated targets of HATs, a number of non-histone proteins have been uncovered as HAT substrates. Aptly, the term 'acetylome' has recently been coined, cataloging all histone acetyltransferase targets (Minucci and Pelicci, 2006). The transcription factor p53 was the first non-histone protein to be identified as a HAT substrate (Gu and Roeder, 1997). Acetylation of the p53 carboxy (C)-terminal DNA binding domain by p300/CBP results in activation of p53 sequence specific DNA binding ability, thereby promoting transcription (Gu and Roeder, 1997). Acetylation also enhances p53 protein stability by competing with MDM2 (mouse double minutes 2) ubiquitylation of lysine residues and proteasomal degradation (Gu and Roeder, 1997; Bode and Dong, 2004 and Glozak *et al.*, 2005). GATA-1, E2F and yin yang 1 are other examples of transcription factors whose DNA binding affinities are regulated by p300/CBP and/or PCAF acetylation (Boyes *et al.*, 1998; Kouzarides, 2000; Martinez-Balbas *et al.*, 2000; Yao *et al.*, 2001; Glozak *et al.*,

2005 and Minucci and Pelicci, 2006). Beyond affecting DNA binding and protein stability, HAT facilitated acetylation of non-histone proteins has also been documented to influence protein-protein interactions, protein dimerization and protein localization (Minucci and Pelicci, 2006 and Lin *et al.*, 2006). For example, PCAF and CBP acetylation of Ku70 disrupts the interaction with Ku70 and the pro-apoptotic protein Bcl2-associated X protein (Bax), allowing Bax translocation to the mitochondria for cytochrome c release and apoptosis (Cohen *et al.*, 2004). Also, p300 mediated acetylation of signal transducer and activator of transcription 3 (STAT3) prompts STAT3 dimerization and subsequent nuclear translocation for DNA binding (Yuan *et al.*, 2005 and Minucci and Pelicci, 2006). Cumulatively, these findings indicate that HATs acetylate many targets beyond exclusively histones and therefore also regulate many processes beyond that of chromatin organization.

### **1.2.3.2. Histone Deacetylases**

In opposition to HAT activity, histone deacetylase (HDAC) enzymes are also involved in gene regulation by catalyzing the removal of acetyl moieties on histone residues and other non-histone proteins (Glozak *et al.*, 2005). By definition HDACs have been largely characterized as transcriptional co-repressors implicated in maintaining a compact chromatin environment inaccessible to DNA binding factors (Bolden *et al.*, 2006). Therefore, it is assumed that the balance between HAT and HDAC activity dictates the overall histone acetylation status and, accordingly, the level of transcriptional competence (de Ruijter *et al.*, 2003). Three diverse classes of HDACs, numbered I through III, have been defined based on homology to the yeast HDACs: reduced potassium dependency 3 (Rpd3), histone deacetylase 1 (Hda1) and silent information regulator 2 (Sir2), in that order (Sengupta and Seto, 2004). Class I and class II HDACs are  $\text{Zn}^{2+}$ -dependent enzymes, which diverge significantly from the Class III Sir2-like HDACs that require nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) for their catalytic activity and are not inhibited by classical histone deacetylase inhibitors (HDIs) (Yang and Seto, 2003). Thus, only the Class I and class II HDACs will serve as the topic of further discussion.

Class I HDACs include the proteins HDAC1, HDAC2, HDAC3 and HDAC8 (Gregorette *et al.*, 2004). Each of these HDACs are ubiquitously expressed and are

characterized by an N-terminal catalytic domain and nuclear localization signal (NLS), which restricts them primarily to the nucleus (Bolden *et al.*, 2006). Class I HDACs, analogous to class II HDACs, utilize their catalytic domain for acetyl removal through two histidine-asparagine charge-relay systems coordinated by a  $\text{Zn}^{2+}$  ion (de Ruijter *et al.*, 2003; Holbert and Marmorstein, 2005 and Lin *et al.*, 2006). However, unaccompanied HDAC1 and HDAC2 are rather inactive deacetylases which require the collaboration of a multi-protein complex to facilitate their deacetylase activity, recruitment and DNA binding ability (de Ruijter *et al.*, 2003). Sin3 (switch independent 3), NuRD (nucleosome remodeling histone deacetylase) and Co-REST (co-repressor to RE1 silencing transcription factor) are three such multi-subunit complexes in which HDAC1 interaction with HDAC2 constitutes the catalytic core (Yang and Seto, 2003). HDAC3 differs somewhat from the other class I HDACs in that it contains both a NLS and a nuclear export signal (NES), and is therefore capable of shuttling between the nucleus and cytoplasm (de Ruijter *et al.*, 2003). Furthermore, HDAC3 has been implicated in other multi-protein complexes with the co-repressors N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoic acid and thyroid hormone receptor), as well as in combination with the class II HDACs, 4, 5 and 7 (Gregorette *et al.*, 2004 and Sengupta and Seto, 2004). HDAC8 is the newest class I member and has yet to be isolated as part of a multi-subunit HDAC complex (Yang and Seto, 2003).

Class II HDACs can be divided into two subclasses; Class IIa, which contains HDAC4, HDAC5, HDAC7 and HDAC9, and Class IIb, formed by HDAC6 and HDAC10 (Verdin *et al.*, 2003; Gregorette *et al.*, 2004 and Bolden *et al.*, 2006). Class II HDACs are characterized by a tissue specific expression pattern, with HDAC4, HDAC5 and HDAC9 observed in heart, skeletal muscle and brain tissues, HDAC7 in primarily the heart and lung, HDAC6 in testis, and HDAC10 in the liver, spleen and kidney (Verdin *et al.*, 2003). The members of the HDAC IIa class are distinguished by a single C-terminal catalytic domain, an NES, an NLS, and an N-terminal region comprised of numerous protein interaction domains (Verdin *et al.*, 2003). Thus, these HDACs are capable of nucleo-cytoplasmic shuttling, and their subcellular localization is largely a determinant of their N-terminal mediated protein interactions (de Ruijter *et al.*, 2003).

Class IIa HDACs have been discovered to interact directly with the transcription factors MEF2 (myocyte enhancer factor 2), BCL6 (B-cell lymphoma 6), and PLZF (promyelocytic leukemia zinc finger), and have been found together in complex with HDAC3 and the nuclear co-repressors N-CoR and SMRT (Yang and Seto, 2003). Class IIb HDACs are classified by the presence of two HDAC domains, and while HDAC6 contains two functional catalytic HDAC domains, HDAC10 has only one functional domain but also contains a vestigial domain (Gregorette *et al.*, 2004). Interestingly, HDAC6 differs significantly from the other class I and class II HDACs since it is primarily localized in the cytoplasm with the microtubule network and deacetylates  $\alpha$ -tubulin (Verdin *et al.*, 2003 and Bolden *et al.*, 2006). HDAC6 has also recently been shown to deacetylate the molecular chaperone heat shock protein 90 (Hsp90) (Kovacs *et al.*, 2005). HDAC10, on the other hand, resides mainly in the nucleus and has been reported to interact with HDAC3 and SMRT (Gregorette *et al.*, 2004). An additional protein, HDAC11, has also been identified (Gao *et al.*, 2002). While HDAC11 remained unclassified for some time, it has now been suggested to be the founding member of a new class of HDACs, class IV (Bolden *et al.*, 2006).

While HDACs have been extensively implicated as transcriptional repressors, a number of recent reports suggest that HDAC activity may be required for gene activation in some cases. The first indication that HDACs may be involved in mechanisms beyond gene repression came from gene expression analysis of HDI treated cells, which drew attention to the fact that HDIs not only altered the expression of a small percentage of genes (2-22%), but both upregulated and downregulated similar proportions of genes. Second, HDAC activity of Hos2, a member of the yeast Rpd3 class I HDACs, was determined to be a requirement for gene activation and an additional report identified that the yeast Rpd3 HDAC was associated with activating osmoresponsive genes (Wang *et al.*, 2002 and De Nadal *et al.*, 2004). Furthermore, STAT5 mediated transcriptional activation was found to require HDAC activity for the recruitment of TBP and RNA polymerase II (Rasche *et al.*, 2003). HDAC activity has also been shown to be necessary for the transcription of many interferon stimulated response genes (Nusinzon and Horvath, 2003; Klampfer *et al.*, 2004; Sakamoto *et al.*, 2004 and Nusinzon and Horvath, 2005). Therefore, in addition to acting as

transcriptional co-repressors, HDACs appear to play a role in the transcriptional activation of particular genes.

#### **1.2.3.3. Other Covalent Histone Modifying Enzymes**

Histone methyl transferases (HMTs) are another important group of histone modifying enzymes, responsible for transferring the methyl group from the donor, S-adenosylmethionine (SAM), to the side chain amino group of lysine and arginine residues on histone H3 and H4 subunits (Santos-Rosa and Caldas, 2005 and Zhang and Dent, 2005). HMTs are capable of mono-, di, or tri- methylation on the epsilon amino group of lysine residues, while the guanidino nitrogens of arginines may only be monomethylated, as well as symmetrically or asymmetrically dimethylated (Shilatifard, 2006). There are three prominent classifications of HMTs: the SET and non-SET domain-containing lysine methyl transferases (KMTs), and the protein arginines methyl transferases (PRMTs) (Shilatifard, 2006). Within the SET domain-containing KMT class, four KMT families are the SUV39 (suppression of position-effect variegation 39), SET1, SET2 and RIZ (retinoblastoma-interacting zinc finger) families, which are characterized by an evolutionarily conserved 130 amino acid catalytic SET (SUV39, Enhancer of zeste and trithorax) domain (Kouzarides, 2002 and Zhang and Dent, 2005). The SET-containing KMTs have been collectively reported to specifically methylate H3 K4, 9, 27, 36 and H4 K20 (Shilatifard, 2006). Presently, Dot1 (disruptor of telomeric silencing 1), which methylates the H3 core at K79, is the only one non-SET domain-containing KMT that has been identified (Shilatifard, 2006).

There are two classes of PRMTs. Class I PRMTs are accountable for catalyzing the asymmetric dimethylation of histone arginine residues, and class II PRMTs catalyze symmetric arginine dimethylation (Kouzarides, 2002). CARM1 (co-activator-associated R-methyltransferase 1) belongs to the class I PRMTs and methylates H3 arginine 2, 17, and 26 (Santos-Rosa and Caldas, 2005). Interestingly, unlike acetylation, site specific histone methylation of lysine and arginine residues dictates different gene regulatory events. For example, methylation of H3 K4 is associated with transcriptionally active genes, while H3 K9 methylated residues recruit HP1 to bring about and sustain a heterochromatin state (Santos-Rosa and Caldas, 2005). CARM1 mediated methylation of H3 R2, 17, and 26 has also been linked with transcriptional activation of nuclear

receptors (Kouzarides, 2002; Santos-Rosa and Caldas, 2005 and Shilatifard, 2006). Therefore, HMTs generate specific histone methylation modifications and regulate an array of processes from gene activation to silencing (Shilatifard, 2006).

Until recently, histone methylation was believed to be a permanent and irreversible histone modification. However, this phenomena appears to be reversible, as a newly discovered enzyme, LSD1 (lysine specific demethylase 1), has been reported to demethylate the mono- or dimethylated forms of H3 K4, but not the trimethylated conformation (Shi *et al.*, 2004 and Bannister and Kouzarides, 2005). Furthermore, within the past year two new demethylases have been characterized, GASC1 (gene amplified in squamous cell carcinoma 1) and JHDM3A (jumonji C-domain-containing histone demethylase 3A), that demethylate di- and trimethylated H3 K9, and trimethylated H3 K9 and K36, respectively (Cloos, *et al.*, 2006 and Klose *et al.*, 2006). As well, a mechanism for the removal of H3 arginine 2, 8, 17 and 26 methylation by deimination has been described, where methylated arginine is converted to citrulline by the enzyme PADI4 (peptidyl arginine deiminase 4) (Bannister and Kouzarides, 2005 and Cuthbert *et al.*, 2004).

Phosphorylation is another important covalent post-translational histone modification that targets histone H1, H2B, H3 and the histone variant H2A.X (Zhang and Dent, 2005). Two such examples of histone phosphorylation include aurora kinase family phosphorylation of H3 S10, which is associated with mitotic chromosome condensation, and phosphatidylinositol 3-kinase-like kinase catalyzed H2A.X S139 phosphorylation, which facilitates DNA repair processes (Santos-Rosa and Caldas, 2005). In addition to acetylation, methylation and phosphorylation, histones may also be modified by ubiquitin and the small ubiquitin-related modifier protein, SUMO (Santos-Rosa and Caldas, 2005). Therefore, histones are substrates for numerous histone modifying enzymes that dictate gene regulatory events.

#### **1.2.4. Histone Deacetylase Inhibitors**

A delicate balance between histone acetylation and histone deacetylation is essential to carry out accurate gene regulatory events within the cell, therefore, it is not surprising that alterations in the balance of HAT and HDAC activity or expression have

been associated with a neoplastic phenotype. For example, HDAC1, HDAC2 and HDAC3 have been reported to be overexpressed in colon cancer, HDAC1 and HDAC2 are upregulated in gastric tumors, and HDAC1 has increased expression levels in hormone refractory prostate carcinoma (Choi *et al.*, 2001; Halkidou *et al.*, 2004; Song *et al.*, 2005; Bolden *et al.*, 2006 and Wilson *et al.*, 2006). In acute promyelocytic leukemia (APL), which is characterized by chromosomal translocations, cells produce fusion proteins of the transcription factor retinoic acid receptor- $\alpha$  (RAR $\alpha$ ) and promyelocytic leukemia (PML) or PLZF protein that facilitate abnormal HDAC recruitment to RAR target promoters and in turn prevent transcription (Minicci and Pelicci, 2006). A fraction of diffuse large-B cell lymphomas display high expression levels of the transcription factor, BCL6. Upon hypoacetylation of BCL6 by HDAC2, the transcription of BCL6-responsive genes is suppressed, including that of cyclin dependent kinase inhibitor p21WAF1 (Bolden *et al.*, 2006). Additionally, CBP mutations that result in a loss of HAT activity have been observed in patients with Rubinstein-Taybi syndrome, who are particularly susceptible to cancer development (Kelly and Marks, 2005). As well, missense mutations of p300 have been reported in colorectal and gastric carcinomas (Muraoka *et al.*, 1996 and Dokmanovic and Marks, 2005). The overall deficit of H4 K16 monoacetylation and H4 K20 trimethylation has also recently been identified as a hallmark of cancer cells (Fraga *et al.*, 2005). Therefore, observations like these have rationalized and intensified the study of histone deacetylase inhibitors (HDIs) as a therapeutic means of restoring the balance between HATs and HDACs in tumor cells.

HDIs have been well documented as promising anti-cancer agents given their ability to selectively induce cell cycle arrest, differentiation and/or apoptosis of cancer cells without affecting normal cells (Marks *et al.*, 2001; Burgess *et al.*, 2004 and Carey and La Thangue, 2006). It is not yet precisely clear how HDIs exert these effects, however, at the structural level, HDIs have been proposed to prevent histone deacetylation activity by binding in the active site pocket and chelating the Zn<sup>2+</sup> ion of class I and class II Zn<sup>2+</sup>-dependent HDACs (Finnin *et al.*, 1999; Somoza *et al.*, 2004 and Vannini *et al.*, 2004). At the molecular level, HDIs were initially predicted to carry out their effects on gene regulation by disrupting the overall balance between histone

deacetylation and acetylation levels within the cell. Therefore, HDIs were hypothesized to bring about a hyperacetylated histone state that enhanced activator and basal transcription factor promoter accessibility, ultimately leading to gene de-repression (Strahl and Allis, 2000). Suitably, this theory was greatly supported for a number of years by the observation that HDIs transcriptionally activated the p21WAF1 promoter, independent of p53 (Nakano *et al.*, 1997). Furthermore, p21WAF1 upregulation was linked to HDI mediated cell cycle arrest, and only strengthened the model that HDIs achieved their anti-tumor effects as a result of histone hyperacetylation and consequent gene activation (Archer *et al.*, 1998). At the present time, HDIs do not appear to act as simplistically as once believed. For instance, early gene profiling experiments indicated that HDIs do not globally activate genes, as predicted, but only specifically alter the expression levels of approximately 2% of genes (Van Lint *et al.*, 1996). In addition, a significant amount of gene profiling work, using DNA microarrays, identified that between 10-22% of genes may be regulated by HDIs (Mariadason *et al.*, 2000 and Peart *et al.*, 2005). It was found that HDIs not only increase gene expression levels but repress a surprisingly equivalent number of gene expression profiles, including those of c-myc cyclin D1 and cyclin A (Lallemant *et al.*, 1996; Nair *et al.*, 2001 and Sasakawa *et al.*, 2003). It was these initial results which provided the basis for the argument that HDIs may elicit their chemo-therapeutic effects by a much broader range of gene regulatory mechanisms than simply histone acetylation.

Over the past few years, evidence has accumulated suggesting that HDIs are capable of altering multiple levels of gene regulation, including stimulating and repressing gene transcription, as well as influencing mRNA stability. Numerous verified examples now exist of genes that are downregulated by HDIs. Recently, it has also been reported that HDIs compromise DNA methyltransferase-3B (DMT3B) and DNA methyltransferase 1 (DMT1) mRNA stability, further suggesting that HDIs may also alter the methylation status of DNA (Xiong *et al.*, 2005 and Januchowski *et al.*, 2007). Still, the mechanisms are unclear as to how HDIs elicit changes in gene expression to ultimately promote cell cycle arrest, apoptosis and/or differentiation of tumor cells. Primarily, the mechanism of non-histone protein acetylation has been examined. HDIs have been proposed to trigger acetylation of non-histone proteins resulting in changes in



the binding affinity of transcription factors, as well as alterations in protein stability, protein dimerization and protein localization (Drummond *et al.*, 2005; Lin *et al.*, 2006 and Minucci and Pelicci, 2006). Accordingly, HDI mediated acetylation of p53 has recently been reported to stabilize p53 protein levels, which in turn stimulates p53-dependent activation of the p21WAF1 and Bax gene promoters (Bolden *et al.*, 2006, Zhao *et al.*, 2006). This ultimately causes an induction of cell cycle arrest and apoptosis, respectively (Bolden *et al.*, 2006 and Lin *et al.*, 2006). Furthermore, the modification of Ku70 acetylation by HDIs initiates Bax release from the Ku70/Bax complex, allowing Bax translocation to the mitochondria for subsequent cytochrome *c* release and apoptosis (Subramanian *et al.*, 2005 and Lin *et al.*, 2006). Histone and non-histone acetylation are, however, likely not the only means of HDI action. Recently, another potential mechanism has been proposed, based on observations that protein phosphatase 1 (PP1) forms complexes with HDAC1, HDAC6 and HDAC10 and that HDIs are capable of disrupting HDAC-PP1 complexes (Brush *et al.*, 2004). HDAC-PP1 complexes have been implicated in preventing the transcription of CREB responsive genes by dephosphorylating the transcription factor CREB, thereby inhibiting subsequent HAT recruitment, histone acetylation and gene activation (Canettieri *et al.*, 2003). HDI mediated disruption of the HDAC-PP1 complexes also leads to CREB phosphorylation and the likely transcriptional activation of target genes, as well as an enhanced association of PP1 with the Akt protein kinase, which mediates Akt dephosphorylation and inactivation (Canettieri *et al.*, 2003 and Chen *et al.*, 2005). Therefore, these findings suggest that HDIs have the potential to alter the phosphorylation pattern of yet unidentified proteins, including histones and transcriptional co-activators, as well as other transcription factors and signaling molecules to selectively regulate gene expression levels and signaling pathways (Alao *et al.*, 2006). Finally, HDIs may mechanistically decrease transcription levels by simply preventing histone deacetylation, such as in the cases of STAT5 and many interferon responsive genes that require HDACs for gene activation (Rasclé *et al.*, 2003 and Nusinzon *et al.*, 2005). Taken together, HDIs elicit their anti-tumor activity through a much broader range of regulatory actions than previously anticipated and highlight the great diversity and complexity by which these drugs function.

#### 1.2.4.1. Histone Deacetylase Inhibitor Classes and Clinical Trials

HDIs can be categorized into four predominant different structural classes: aliphatic acids, hydroxamic acids, benzamides and cyclic peptides (Garcia-Manero and Issa, 2005; Bolden *et al.*, 2006 and Minucci and Pelicci, 2006) (Table 1.1.). Interestingly, a number of these compounds have been reported to exhibit both chemo-preventive and chemotherapeutic activity in a range of human cancer cell lines *in vitro* and tumor bearing models *in vivo* (Marks *et al.*, 2001; Vigushin and Coombes, 2002 and Arts *et al.*, 2003). Butyrate was the first discovered HDI and belongs to the aliphatic acid class of HDIs. This compound has been found to reside naturally in the colonic lumen as a product of anaerobic bacterial fermentation of dietary fibers (Roediger, 1980). Along with butyrate, acetate and propionate are also generated from the microbial fermentation process, but only propionate has weak HDI activity (Cousens *et al.*, 1979). Butyrate is a non-competitive inhibitor of HDACs and requires millimolar concentrations to effectively inhibit histone deacetylation, which are physiologically realistic in the colon, as butyrate concentrations in the range of approximately 12 - 24 mM have been detected in the human adult feces (Topping and Clifton, 2001 and Davie, 2003). Besides acting as a HDI, butyrate also exhibits pleiotropic effects on the cytoskeleton and alters protein phosphorylation and DNA methylation levels within the cell (Kruh, 1982; Sowa *et al.*, 2000; Vigushin and Coombes, 2002 and Arts *et al.*, 2003). Importantly, butyrate serves as the primary energy source of colonocytes and plays an essential role in maintaining the homeostatic balance and growth of normal colonic epithelial cells (Roediger, 1980). However, butyrate also displays anti-proliferative effects and has been documented to stimulate cell cycle arrest, differentiation and apoptosis in tumor cells, while normal cells show little sensitivity (Carey and La Thangue, 2006). Therefore, butyrate has been implicated as a chemo-preventive agent against the progression of colorectal tumorigenesis, further strengthening the argument that high fiber diets may decrease colorectal cancer incidence (Burkitt, 1971 and Sowa and Sakai, 2000).

Sodium phenylbutyrate has been clinically approved for use in the management of urea cycle disorders since 1996 and was the first HDI to be investigated as a chemotherapeutic agent in clinical trials. However, as an anti-tumor agent, butyrate displayed

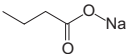
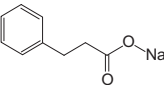
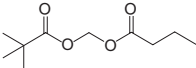
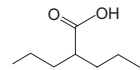
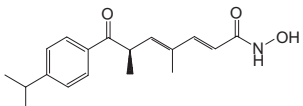
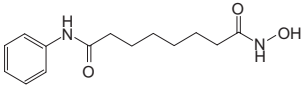
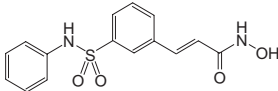
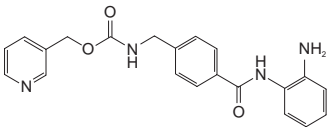
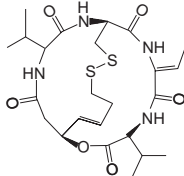
Class	Name	Structure	Current Clinical Trial Status
Aliphatic acid	Sodium Butyrate		—
	Sodium phenylbutyrate		Phase I
	Pivaloyloxymethyl butyrate (AN-9)		Phase II
	Valproic acid		Phase I
Hydroxamic acid	Trichostatin A (TSA)		—
	Suberoyl anilide hydroxamic acid (SAHA)		Phase II
	PXD-101		Phase II
Benzamide	MS-275		Phase II
Cyclic peptide	Depsipeptide (FK-228)		Phase II

Table 1.1. Histone deacetylase inhibitors, classes, structures, status of clinical trials. (Minucci *et al.* 2006, Inche *et al.* 2006, Dokmanovic *et al.* 2005, Garcia-Manero *et al.* 2005)

limited clinical utility owing to its rapid clearance rate, low potency and lack of specificity (Vigushin and Coombes, 2002 and Rosato and Grant, 2004). Despite this, there were a couple of notable observations derived from butyrate studies, such as a partial remission of a child with acute myelogenous leukemia (AML) treated with sodium butyrate, and complete clinical remission of a young patient with relapsed APL for a seven month period when treated in combination with all-trans-retinoic acid (ATRA) and sodium phenylbutyrate (Novogrodsky *et al.*, 1983 and Warrell *et al.*, 1998). These observations suggested that a more potent and specific HDI may be of clinical value. Currently, pivaloyloxymethyl butyrate (AN-9) and valproic acid are two different HDIs that belong to the aliphatic acid class that are going through clinical trials (Table 1.1). AN-9 is a butyrate prodrug, which is hydrolyzed to form pivalic acid, formaldehyde and butyrate once it has been transported into the cell (Patnaik *et al.*, 2002). When compared to butyrate, AN-9 has 10-fold greater potency and reduces tumor growth in human breast, ovarian, colorectal and non-small cell lung carcinomas to a much greater degree (Patnaik *et al.*, 2002). Phase II clinical trials of AN-9 administered in forty seven advanced non-small cell lung cancer patients have provided promising results, with three patients going into partial remission and fourteen other patients encountering cancer stabilization for 12 weeks or more (Reid *et al.*, 2004). Valproic acid is an anticonvulsant drug that has been clinically used for over a decade, but has only recently been identified as a HDI (Rosato and Grant, 2004). Interestingly, valproic acid has a much longer half-life than butyrate, appears to function as a competitive inhibitor of HDACs, and has been documented to induce growth inhibition, differentiation and apoptosis of leukemia cells *in vitro* (Garcia-Manero and Issa, 2005). Furthermore, valproic acid has a well documented history of potential side effects, making it an appealing drug to study as a potential anti-cancer agent (Rosato and Grant, 2004). Phase I clinical trials have generated compelling results, as 30% of older patients with AML or myelodysplastic syndrome displayed clinical improvement upon treatment with valproic acid (Pilatrino *et al.*, 2005 and Minucci and Pelicci, 2006). Currently, a new formulation of valproic acid, named Savicol, is in phase II trials for the treatment of Familial Adenomatous Polyposis, an inherited colorectal cancer disorder (Garber, 2007).

Trichostatin A (TSA) is the original member of the hydroxamic acid class of HDIs and was initially isolated from the antifungal antibiotic *Streptomyces hygroscopicus* (Yoshida *et al.*, 1990) (Table 1.1). This compound is a reversible and specific inhibitor of class I and class II HDACs, directly interacting with the  $\text{Zn}^{2+}$  ion at the bottom of the HDAC catalytic pocket (Somoza *et al.*, 2004 and Vannini *et al.*, 2004). Furthermore, TSA is significantly more potent than butyrate, inducing cell cycle arrest, differentiation and apoptosis in tumor cells at micromolar and nanomolar concentrations rather than the millimolar concentrations of butyrate (Rosato and Grant, 2004). However, due to high cellular toxicity, TSA has not entered clinical trials (Lin *et al.*, 2006). Suberoylanilide hydroxamic acid (SAHA) is a synthetic inhibitor from the hydroxamic acid class that has fared notably well in clinical trials featuring patients with solid and hematological tumors (Table 1.1). Decreases in tumor size have been experienced in patients with non-Hodgkin's lymphoma, Hodgkin's disease, mesothelioma, bladder, thyroid, renal and laryngeal cancers. Partial remissions have also been accrued in five patients with relapsed T-cell lymphoma and one complete clinical response has been reported in a patient with AML (Garcia-Manero and Issa, 2005 and Minucci and Pelicci, 2006). Among concerns with SAHA administration is the relatively short half-life and list of side effects, including dehydration, fatigue, diarrhea, nausea, anorexia, thrombocytopenia and anemia, which are all completely reversed upon SAHA removal (Garcia-Manero and Issa, 2005; Monneret, 2005 and Minucci and Pelicci, 2006). Excitingly, in October of 2006 SAHA was approved by the Food and Drug Administration (FDA) as the first HDI anti-cancer drug for the treatment of cutaneous T-cell lymphoma (CTCL), a rare type of non-Hodgkin's disease (Garber, 2007 and Marks and Breslow, 2007). In addition to SAHA, other next generation hydroxamic acids have since been designed in an attempt to enhance retention times and minimize side effects. One of these, PXD101, is currently being evaluated in phase II clinical trials as a treatment for multiple myeloma and T-cell lymphomas, as well as ovarian and colorectal cancer (Monneret, 2005) (Table 1.1).

The benzamide and cyclic peptide classes also have HDI compounds in clinical trials. MS-275 is a synthetic benzamide derivative that has a particularly long retention time and requires micromolar concentrations to stimulate cell cycle arrest,

differentiation and apoptosis in tumor cells (Minucci and Pelicci, 2006 and Garber, 2007) (Table 1.1). Currently, MS-275 is undergoing phase II clinical trials for AML. Depsipeptide (FK228) is a natural polypeptide that was initially isolated from *Chromobacterium violaceum* (Monneret, 2005) (Table 1.1). FK-228, like AN-9, functions as a HDI prodrug, and contains an intramolecular disulfide bond that is reduced by glutathione to produce its active form, redFK, upon entering the cell (Garcia-Manero and Issa, 2005; Monneret, 2005 and Lin *et al.*, 2006). FK-228 is a potent HDI prodrug and requires only nanomolar concentrations to inhibit HDACs and cause anti-tumor effects in neoplastic cells (Rosato and Grant, 2004 and Garber, 2007). In phase II clinical trials, FK-228 has demonstrated partial and complete clinical responses in as many as 57% of patients with T-cell lymphomas, warranting possible clinical approval (Minucci and Pelicci, 2006). An application for FDA authorization is expected to be filed in 2007 for FK-228 treatment of CTCL and peripheral T-cell lymphoma (PTCL), although some clinical skepticism exists over the use of this drug given its past association with cardiac side effects (Minucci and Pelicci, 2006 and Garber, 2007).

While HDIs have primarily been discussed as monotherapies in clinical trials, it is important to note that a number of clinical trials are ongoing to investigate the role of HDIs in combination with many other compounds including differentiation agents (ATRA, phorbol myristate acetate), DNA demethylating agents (5-aza-2'-deoxycytidine, 5-azacytidine), cyclin dependent kinase inhibitors (Flavopiridol), Hsp90 antagonists (17-AAG), proteasome inhibitors (Bortezomib), angiogenesis inhibitors (Avastin), and other approved chemotherapeutic drugs (Taxol, Herceptin, doxorubicin, cisplatin, fludarabine, fluorouracil) (Rosato and Grant, 2004; Lin *et al.*, 2006; Minucci and Pelicci, 2006 and Garber, 2007). In addition, a great deal of work is currently focused on the identification of HDAC isozyme specific inhibitors, given that SAHA and other HDIs in clinical trials are broad range inhibitors of both class I and class II HDACs (butyrate, valproic acid, TSA, SAHA, PXD101), or purely the class I HDACs (MS-275, FK-228) (Bolden *et al.*, 2006 and Garber, 2007). One such example of an isoform specific HDI exists for HDAC6, named tubacin, which exclusively prevents  $\alpha$ -tubulin deacetylation (Haggarty *et al.*, 2003). Taken together, a number of HDIs have

presented encouraging clinical trial results for treatment of hematological and solid malignancies, both alone and in combination with other chemotherapeutic agents, meriting further study of the mechanisms associated with HDI mediated induction of cell cycle arrest, differentiation and apoptosis. Furthermore, it is likely that the recent FDA approval of SAHA as an anti-cancer drug will only serve to further intensify the study of this rapidly developing field.

### **1.3. Eukaryotic Transcriptional Regulation**

The expression of genes is a tightly regulated process, centrally controlled by transcription, which facilitates normal cellular growth, development and survival (Saunders *et al.*, 2006). Gene transcription is characterized by six cyclic stages: pre-initiation complex assembly, open complex arrangement, transcriptional initiation, promoter clearance, transcript elongation and termination (Sims *et al.*, 2004 and Svejstrup, 2004). However, chromatin structure regulates transcription even before pre-initiation complex (PIC) assembly. This is best exemplified by the repressive state that associated nucleosomes impose on promoter DNA, largely as a consequence of promoter site inaccessibility for the transcriptional machinery and RNA polymerase II (Pol II), which transcribes all protein encoding genes in eukaryotes (Boeger *et al.*, 2005).

#### **1.3.1. Promoter Architecture**

There are numerous *cis*-regulatory DNA elements present in eukaryotic promoters which recruit *trans*-acting factors to cooperate in the direction of Pol II mediated transcription (Lemon and Tjian, 2000). Generally, *cis*-acting elements that control Pol II transcription include core promoter, proximal promoter, enhancer, silencer and boundary/insulator elements (Butler and Kadonaga, 2002; Kadonaga, 2002 and Szutorisz *et al.*, 2005) (Figure 1.1.). The core promoter resides within 40 bp upstream and downstream of the transcription start site, may contain a core promoter element and represents the minimal DNA sequence necessary for the correct assembly and orientation of the PIC for Pol II driven transcription (Butler and Kadonaga, 2002; Szutorisz *et al.*, 2005; Thomas and Chiang, 2006 and Yang *et al.*, 2007). The proximal

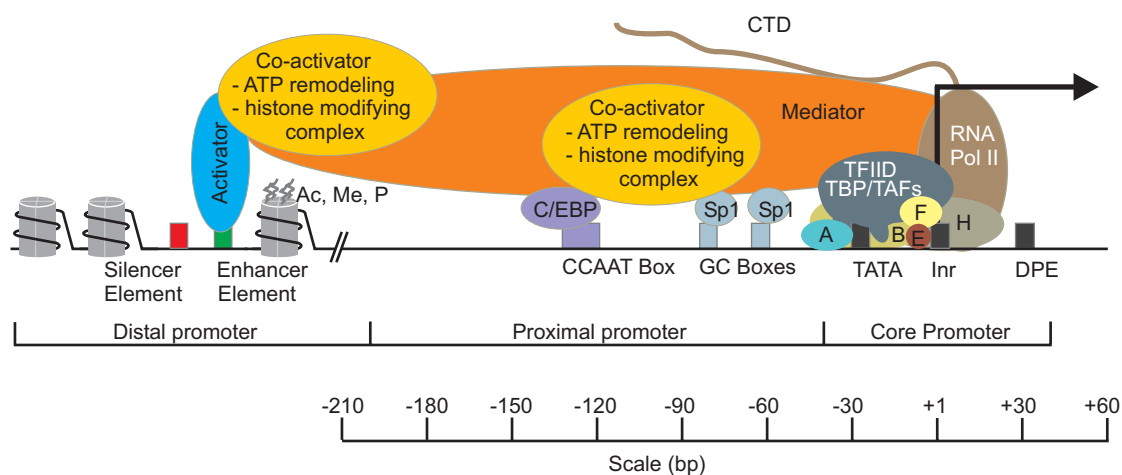


Figure 1.1. Eukaryotic promoter architecture and components of the RNA Polymerase II transcriptional machinery. The regions of the distal, proximal and core promoters are outlined. Silencer and Enhancer elements reside in the distal promoter. Transcription factor binding sites are present in the proximal promoter, the TATA box, Inr and DPE are shown in the core promoter. The factors necessary to activate transcription are displayed, including activator and co-activator complexes, transcription factors, GTFs - TFIID, TFIIA (A), TFIIB (B), TFIIF (F), TFIIE (E), TFIIH (H), RNA Pol II and Mediator. Potential post-translational histone modifications are denoted as Ac (acetylation), Me (methylation), P (phosphorylation). (Boeger *et al.*, 2005, Thomas and Chiang, 2006)



promoter is located between 40 and 200 bp upstream of the transcription start site and houses proximal promoter elements or transcription factor binding sites that associate with *trans*-activating factors and serve to boost promoter activity (Kadonaga, 2002 and Szutorisz *et al.*, 2005). Distal enhancer elements stimulate transcription of associated promoters via the recruitment of specific activators to DNA recognition sites, regardless of distance or location (either upstream or downstream) from the transcription start site (Blackwood and Kadonaga, 1998). Silencer elements recruit repressor proteins to inhibit transcription over long distances and boundary or insulator elements block the effects of enhancers and silencers (Butler and Kadonaga, 2002).

#### **1.3.1.1. The Core Promoter Element**

Currently, seven core promoter elements have been elucidated, termed the TATA box, initiator (Inr) element, downstream promoter element (DPE), motif ten element, downstream core element, upstream TFIIB-recognition element (<sup>u</sup>BRE), downstream TFIIB-recognition element (<sup>d</sup>BRE) and X core promoter element 1 (Thomas and Chiang, 2006 and Tokusumi *et al.*, 2007). Of these, the most widely studied are the TATA box and Inr elements. The TATA box element is characteristically located 25 – 30 bp upstream of the transcription start site, is recognized by the consensus sequence TATA(A/T)A(A/T)(A/G) and bound by the TATA-binding protein (TBP) subunit of TFIID (transcription factor for RNA polymerase II), the core component of PIC assembly (Thomas and Chiang, 2006). Conversely, the Inr element covers the transcriptional start site, has the consensus sequence PyPyA<sup>+</sup>N(T/A)PyPy, is found in both TATA-containing and TATA-less promoters and is recognized by the TBP-associated factor (TAF) subunits of the TFIID, TAF1 and TAF2 (Chalkley and Verrijzer, 1999 and Thomas and Chiang, 2006). Interestingly, 24% of human genes hold a TATA box-like element but only 10% have a consensus TATA box (Yang *et al.*, 2007). Moreover, 16% of human promoters contain an Inr element in combination with a TATA-box element and 30% possess only an Inr element (Yang *et al.*, 2007). In addition to TATA-box and Inr elements, the DPE, located between 28 to 34 bp downstream of the start site, associates with TAF6 and TAF9 of the TFIID complex (Burke and Kadonaga, 1997 and Shao *et al.*, 2005). The BREs, residing at -38 to -32 bp (<sup>u</sup>BRE) and -23 to -27 bp (<sup>d</sup>BRE) upstream of the start

site in some promoters bind the general transcription factor (GTF), TFIIB (Lagrange *et al.*, 1998 and Thomas and Chiang, 2006). Thus, a number of diverse core promoter elements provide a platform for the recognition and collection of the RNA Pol II multi-protein PIC.

#### **1.3.1.2. Proximal Promoter and Enhancer Elements**

Often preliminary to PIC formation, activator binding occurs at either exposed, nucleosome free, distal enhancer elements or proximal promoter elements (Szutorisz *et al.*, 2005). Examples of two common proximal promoter elements include GC boxes that associate with the specificity protein (Sp) family of transcription factors and CCAAT boxes bound by the CCAAT/enhancer binding protein (C/EBP) family, which are present in 97% and 64% of potential human proximal promoter regions, respectively (Suzuki *et al.*, 2001). Activators bound to enhancer and proximal promoter elements regularly recruit co-activator complexes, including ATP-dependent chromatin remodeling complexes that mediate nucleosome movement and /or histone removal, and histone modifying enzyme complexes that introduce covalent post-translational modifications on the N-terminal tails of histones (Li *et al.*, 2004b). Such modifications may locally decondense chromatin associated with promoter DNA, or establish docking sites for further co-activator or transcription factor recruitment. Taken together, co-activators enhance promoter DNA availability by uncovering *cis*-regulatory elements, including core promoter elements, and thereby facilitate TFIID binding and PIC complex assembly (Szutorisz *et al.*, 2005). A “facilitated tracking mechanism” has been proposed to describe enhancer involvement in transcriptional activation (Blackwood and Kadonaga, 1998 and Hatzis and Talianidis, 2002). In this model, enhancer bound activator and co-activator complexes track along the DNA progressively remodeling nucleosomes and chromatin. Once the complex reaches the core promoter it is proposed that a stable looped structure is formed at the promoter that aids in transcriptional activation. Furthermore, activators also recruit the multi-protein co-activator complex Mediator, which communicates regulatory signals to the PIC (Jones and Kadonaga, 2000). Therefore, transcriptional regulation is fundamentally dependent on *cis*-acting promoter elements, and their interaction with *trans*-acting factors collaborates to define the net activation potential of a given promoter.

### **1.3.2. Pre-initiation and the RNA Polymerase II Transcriptional Machinery**

Following nucleosome remodeling or accessibility of core promoter element, the first phase of transcription is characterized by formation of the PIC. The PIC is composed of six GTFs (TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, TFIIH), and RNA polymerase II (Boeger *et al.*, 2005 and Klug, 2005) (Figure 1.1.). In addition to the PIC, a multi-subunit co-activator complex Mediator is necessary for activator mediated Pol II transcription (Szutorisz *et al.*, 2005).

#### **1.3.2.1. Pre-initiation Complex Assembly and Open Complex Arrangement**

Intensive efforts have gone into understanding the assembly pattern of the PIC in Pol II directed transcription, given that accurate formation of the PIC culminates in RNA synthesis. Two models describing PIC assembly have been proposed: a sequential assembly model and a preassembly or holoenzyme model. In the sequential assembly model, GTFs gather at the core promoter in a structured manner, such that TFIID binds the TATA box or core promoter, followed by the stepwise recruitment of TFIIA, TFIIB, Pol II/TFIIIF, TFIIE and TFIIH to activate transcription (Lemon and Tjian, 2000 and Thomas and Chiang, 2006). Conversely, in the holoenzyme model, TFIID and TFIIA associate with the core promoter prior to the recruitment of a large preassembled complex containing Pol II, TFIIB, TFIIE, TFIIIF, TFIIH, and possibly other chromatin remodeling factors, to stimulate promoter activation (Lemon and Tjian, 2000 and Thomas and Chiang, 2006). While neither model has yet to be definitively confirmed, the sequential model appears to be favored at this time (Lemon and Tjian, 2000 and Thomas and Chiang, 2006).

Regardless of the pattern of PIC assembly, TFIID is the GTF that facilitates interaction with the core promoter (Figure 1.1.). TFIID is a large, multi-subunit complex composed of TBP and approximately 13 TAFs (Thomas and Chiang, 2006). The TBP subunit of TFIID promotes the recognition of TATA box promoters, directly interacting with the TATA box and stimulating DNA bending around the core promoter element (Burley and Roeder, 1996 and Woychik and Hampsey, 2002). Furthermore, a group of TFIID TAF subunits also play a role in promoter recognition, as identification of Inr elements arises from TAF1-TAF2 complexes and TAF6-TAF9 recognizes DPEs (Chalkley Verrijzer, 1999 and Lagrange *et al.*, 1998). TFIID is, therefore, central to

promoter site detection. However, TFIID also has co-activator capability, largely due to the presence of TAF1 (Thomas and Chiang, 2006).

TAF1 is an essential protein in yeast, fruit flies, and hamster cell lines and is responsible for the regulation of approximately 30% and 18% of genes in yeast and hamster cells, respectively (Holstege *et al.*, 1998 and O'Brien and Tjian, 2000). Furthermore, TAF1 is a multidimensional protein capable of interacting with a number of activator proteins including jun, RB, MDM2 and cyclin D, and other GTFs such as TFIIA, TFIIE and TFIIIF (Wassarman and Sauer, 2001). As well, TAF1 binds acetylated histone H3 K14 and histone H4 K5, K8, K12, K16 residues via its two bromodomains and also yields acetyltransferase (AT) and kinase activities (Wassarman and Sauer, 2001 and Thomas and Chiang, 2006) (Figure 1.2.). Hence TAF1 endows TFIID with co-activator function by linking activators to the PIC, as well as the ability to modify histones, GTFs and other components of the Pol II transcription complex. Taken together, TFIID functions both in core promoter element recognition by serving as the platform for PIC assembly, and as a transcriptional co-activator.

Following TFIID recruitment to the core promoter, TFIIA may associate with the promoter to stabilize the interaction between TBP and the TATA box by binding both TBP and the DNA upstream of the TATA box (Thomas and Chiang, 2006). However, TFIIA does not appear to be required for the transcription of all genes (Thomas and Chiang, 2006). TFIIB, on the other hand, is an indispensable component of the PIC. As an individual polypeptide, the C-terminal or core domain of TFIIB interacts with sequences both upstream and downstream of the TATA box, as well as with BREs (Woychik and Hampsey, 2002 and Bushnell *et al.*, 2004). Association of TFIIB with the TATA-TFIID complex further stabilizes the formation of the bent DNA, thereby inhibiting TBP loss, and provides a docking site within the N-terminal zinc binding domain of TFIIB for Pol II entry into the PIC (Bushnell *et al.*, 2004). The twelve subunit Pol II catalytic enzyme contacts TFIIB near the RNA exit channel of the polymerase, via its Rpb1 and Rpb2 subunits, and concurrently draws TFIIIF into the PIC (Thomas and Chiang, 2006). TFIIB promotes RNA Pol II recognition of the transcription start site, facilitating the entry of downstream DNA into the polymerase active center (Boeger *et al.*, 2005 and Thomas and Chiang, 2006). TFIIIF is a

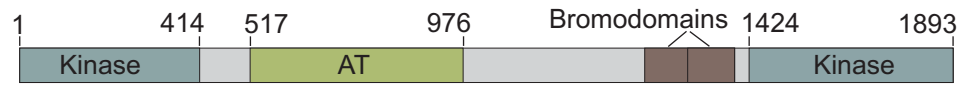


Figure 1.2. The protein structure of TAF1. The enzymatic domains of TAF1 include N-terminal and C-terminal kinase domains and an acetyl-transferase (AT) domain. The two tandem bromodomains of TAF1 are also outlined. (Wassarman *et al.*, 2001)

heterotetramer that associates with Pol II, and is composed of two subunits of serine-threonine kinase RNA polymerase II-associated protein 74 (RAP74) and two subunits of RNA polymerase II-associated protein 30 (RAP30) (Woychik and Hampsey, 2002). TFIIF encourages RNA Pol II recruitment to TFIIB associated PICs, interacts with DNA sequences on both sides of the TATA box, assists in selection of the transcription start site, stabilizes the TATA-TFIID-TFIIB complex, and drafts both TFIIE and TFIIH to the PIC (Woychik and Hampsey, 2002 and Thomas and Chiang, 2006). Accordingly, the heterotetramer TFIIE, made up of two TFIIE $\alpha$  and two TFIIE $\beta$  subunits, interacts with the promoter DNA, TFIIB, Pol II and TFIIF to recruit TFIIH to the PIC (Boeger *et al.*, 2005 and Thomas and Chiang, 2006). TFIIH is the biggest and sole GTF with enzymatic activities (Woychik and Hampsey, 2002). The ten subunits that comprise TFIIH include: two ATPase DNA helicases, gene defective in xeroderma pigmentosum patients complementation group B (p89/XPB) and gene defective in xeroderma pigmentosum patients complementation group D (p80/XPD), p62, p52, p44, p40/CDK7, p38/Cyclin H, p34, p32/MAT1 and TFB5 (Thomas and Chiang, 2006). The ATPase/helicase activity of TFIIH triggers DNA unwinding of approximately 11-15 bp around the transcription start site, forming an open complex (Saunders *et al.*, 2006). A single stranded DNA template then enters the active center of Pol II while the non-template strand is confined by TFIIF (Bushnell *et al.*, 2004 and Boeger *et al.*, 2005). Accurate assembly of GTFs and Pol II into a PIC is proficient for the initiation of *in vitro* basal transcription, however, the presence of the Mediator co-activator complex, enhances basal transcription levels and is necessary to activate transcription in response to activators (Kornberg, 2005 and Malik and Roeder, 2005).

#### **1.3.2.2. Mediator**

Mediator is a multi-protein co-activator complex which communicates regulatory signals from enhancer and proximal promoter elements to the PIC to either activate or repress transcription. As such, it is considered an essential constituent of the Pol II transcriptional machinery (Kornberg, 2005) (Figure 1.1). Mediator was initially isolated from *Saccharomyces cerevisiae* as a 20 subunit complex and has since been found to interact with the activation domains of transcription factors as well as with Pol II (Conaway *et al.*, 2005). Exploitation of activator – Mediator associations has led to

the identification of various mammalian Mediator counterparts, such as thyroid hormone receptor-associated proteins/SRB (suppressors of RNA pol B) - Med containing cofactor (TRAP/SMCC), vitamin D receptor-interacting protein (DRIP), and cofactor required for Sp1 activation (CRSP) (Conaway *et al.*, 2005). However, a unifying mechanism of Mediator recruitment to the region of the promoter, as well as the process by which Mediator is able to connect activators to the PIC remains to be elucidated (Conaway *et al.*, 2005; Kornberg, 2005; Malik and Roeder, 2005 and Roeder, 2005).

### **1.3.3. Transcription Initiation and Promoter Clearance**

Assembly of the transcriptional machinery (PIC and Mediator) poises RNA polymerase II for initiation, while the ATPase / helicase activity of the p89/XBP subunit of TFIIF initiates ribonucleotide synthesis by melting the double stranded DNA and promoting entry of the template strand into the active center of Pol II (Sims *et al.*, 2004 and Saunders *et al.*, 2006). This generates an initially transcribing complex, however, Pol II must break away from the core promoter and some of the general transcriptional machinery before promoter clearance occurs and transcript elongation ensues (Saunders *et al.*, 2006). Promoter clearance, or the transition from an initially transcribing complex into an early elongation complex, is characterized by the rivalry between TFIIB and the synthesizing RNA transcript for the Pol II active center and exit channel. Early in transcription, synthesis of the first 5 bp of RNA is stabilized by the interaction of the TFIIB N terminus with the Pol II active site that occurs through the RNA exit channel (Bushnell *et al.*, 2004 and Boeger *et al.*, 2005). Continued ribonucleotide synthesis, therefore, eventually forces a competition between TFIIB and a 9 bp RNA transcript for the Pol II active center and departure from the Pol II exit channel (Bushnell *et al.*, 2004 and Boeger *et al.*, 2005). If the transcript is unable to overtake TFIIB, abortive transcription occurs, and a new round of transcription must be initiated. However, if the RNA prevails, the initially transcribing complex clears the promoter and transitions into an early elongating complex (Bushnell *et al.*, 2004; Boeger *et al.*, 2005 and Saunders *et al.*, 2006). Furthermore, a subsequent round of transcription can be initiated at the core promoter, following promoter clearance (Sims *et al.*, 2004).

Pol II also plays a fundamental role in the maturation of the initiating complex into an early elongating complex. The largest subunit of Pol II, Rpb1, has a large unstructured carboxy-terminal domain (CTD), consisting of 52 tandem consensus heptapeptide repeats of Y<sub>1</sub>S<sub>2</sub>P<sub>3</sub>T<sub>4</sub>S<sub>5</sub>P<sub>6</sub>S<sub>7</sub>, which may be phosphorylated, glycosylated, ubiquitylated, or undergo *cis/trans* proline isomerization (Meinhart *et al.*, 2005; Phatnani and Greenleaf, 2006; Saunders *et al.*, 2006 and Thomas and Chiang, 2006). Interestingly, the phosphorylation status of Ser 2 and Ser 5 residues within the CTD heptapeptide repeats is representative of the transcriptional stage: the CTD of Pol II assembled into the PIC is hypophosphorylated (Pol IIA), while the elongating Pol II is hyperphosphorylated (Pol IIO) (Sims *et al.*, 2004 and Saunders *et al.*, 2006). In particular, Ser 5 phosphorylation elevates in association with promoter clearance but decreases in the 3' region of the gene, while Ser 2 phosphorylation predominates during productive elongation toward the 3' of the gene (Svejstrup, 2004 and Phatnani and Greenleaf, 2006). Ser 5 phosphorylation within the CTD is carried out by the TFIIF subcomplex of CDK7/Cyclin H and MAT1, the kinase activity of which is significantly enhanced by Mediator association (Svejstrup, 2004 and Meinhart *et al.*, 2005). The mechanism by which Ser 5 phosphorylation facilitates promoter clearance and conversion into an early elongating complex is unclear, however, it has been hypothesized that phosphorylation of the CTD may result in the disruption of Pol II contacts with the PIC and, possibly, Mediator (Svejstrup, 2004). In addition, phosphorylated Ser 5 also behaves as a platform for the recruitment of mRNA capping enzymes (Phatnani and Greenleaf, 2006).

#### **1.3.4. Transcript Elongation**

##### **1.3.4.1. From the Early Elongation Complex to Productive Elongation**

The early elongating complex is still a slightly unstable complex that may undergo transcript slippage, backtracking, and arrest within the first 30 nucleotides of RNA synthesis (Saunders *et al.*, 2006). It is therefore the penultimate complex en route to the development of a mature elongating complex. During or following transcript initiation, DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) sensitivity inducing factor (DSIF), composed of a suppressor of Ty 4 (SPT4) and 5 (SPT5) subunit, interacts



with Pol II (Orphanides and Reinberg, 2002 and Sims *et al.*, 2004). The negative elongation factor (NELF), which is subsequently recruited to the early elongating complex, cooperates with DSIF to stimulate an early Pol II pause between +20 to +40. This has been hypothesized to coincide with the capping of the emerging nascent RNA, and may serve as a fundamental checkpoint for an adequately equipped Pol II complex or a properly capped RNA prior to productive elongation (Orphanides and Reinberg, 2002; Shilatifard, 2004; Sims *et al.*, 2004; Svejstrup, 2004 and Saunders *et al.*, 2006). A commitment to productive elongation is facilitated by the kinase activity of positive transcription elongation factor b (P-TEFb), which is comprised of Cdk9 and one of cyclin T1, cyclin T2a, cyclin T2b or cyclin K (Shilatifard, 2004 and Sims *et al.*, 2004). P-TEFb phosphorylates the SPT5 subunit of DSIF and Ser 2 of the Pol II CTD, leading to NELF dissociation and relief of the Pol II pause (Shilatifard, 2004 and Sims *et al.*, 2004). In addition, the TFIIS elongation factor alleviates the elongation pause by realigning the Pol II active center with the correct 3'-OH of the RNA transcript upon backtracking (Sims *et al.*, 2004). TFIIF also re-associates with the paused Pol II complex to reactivate elongation (Saunders *et al.*, 2006).

Throughout productive elongation, the Pol II CTD remains phosphorylated at Ser 2. The rate of elongation is stimulated by TFIIS and TFIIF, as well as members of the eleven-nineteen lysine-rich in leukemia (ELL) and Elongin families (Shilatifard *et al.*, 2003). Similar to TFIIF, the ELL and Elongin family members bear a repressive effect on transient Pol II transcriptional pausing and are, therefore, important enhancers of elongation (Shilatifard, 2004 and Sims *et al.*, 2004). In addition, accessory elongation factors allow Pol II to transverse chromatin during transcript elongation. One such factor, known as facilitates chromatin transcription (FACT), interacts with H2A-HAB dimers to promote nucleosome destabilization by loss of a single H2A-H2B dimer, and subsequent nucleosome reassembly following Pol II passage (Reinberg and Sims, 2006 and Workman, 2006).

### **1.3.5. Termination**

Transcript termination occurs when Pol II is released from the DNA template. Accurate termination of transcription is imperative for the induction of gene expression

levels. Distinct from transcription initiation, however, termination is not defined by consensus terminator sequences (Svejstrup, 2004). But is tightly linked to mRNA polyadenylation, and will be discussed in further detail within the following section.

Cumulatively, this section highlights the multiple levels in the transcriptional process at which gene expression is regulated; from the chromatin template, to the assembly of the PIC, transcriptional initiation, promoter clearance, the transition from early elongation to productive elongation and precise termination.

## **1.4. mRNA**

### **1.4.1. Co-transcriptional mRNA Processing Events**

A newly synthesized RNA transcript is not readily amenable for nuclear export and translation into a protein. Rather, a series of processing events are required to generate a mature mRNA, including the addition of a 5' cap structure, intron excision of the precursor-mRNA (pre-mRNA), and the acquisition of a 3'-polyadenylated (poly(A)) tail (Orphanides and Reinberg, 2002 and Proudfoot *et al.*, 2002). A considerable amount of evidence suggests that most nascent RNA transcript processing occurs as Pol II traverses the gene, in a co-transcriptional or coupled manner. The CTD of Pol II is imperative to pre-mRNA processing, as deletion of the CTD has been shown to obstruct capping, splicing and polyadenylation site cleavage events (McCracken *et al.*, 1997a; McCracken *et al.*, 1997b; Fong and Bentley, 2001 and Zoria and Bentley, 2004). Fundamentally, the CTD acts as a platform for the recruitment of various pre-mRNA processing factors throughout the transcription cycle and, in return, the execution of processing events may influence Pol II directed transcription (Neugebauer, 2002).

#### **1.4.1.1. Addition of the 5' Cap Structure at the Early Elongation Pause**

The addition of a 5' 7-methyl-guanosine cap is the first processing reaction carried out on a Pol II synthesized pre-mRNA. The reaction proceeds following the synthesis of approximately 20 to 40 nucleotides and nascent RNA departure from the exit channel of the Pol II enzyme. Three enzymes are responsible for the formation of the cap (Shatkin and Manley, 2000; Proudfoot *et al.*, 2002; Shilatifard *et al.*, 2003 and Gu and Lima, 2005). First, a RNA 5'-triphosphatase (RT) enzyme removes the  $\gamma$ -

phosphate from the first nucleotide of the pre-mRNA transcript to generate a diphosphate. This reaction is followed by the action of RNA guanylyltransferase (GT), which transfers a GMP cap to the diphosphate nucleotide at the beginning of the chain in a 5'-5' triphosphate linkage. Finally, RNA N7 guanine methyltransferase (MT) shifts a methyl group from SAM to the N7 position on the GMP moiety to ultimately form a methyl-7-guanosine(5')ppp(5')N cap on the pre-mRNA. In mammalian cells, the RT and GT capping enzymes reside in a single bifunctional polypeptide (Zorio and Bentley, 2004 and Sims *et al.*, 2004).

The phosphorylated form of the Pol II CTD interacts directly with the GT and MT capping enzymes. Importantly, TFIIH mediated phosphorylation of Ser 5 residues within the CTD stimulates GT activity (Cho *et al.*, 1997 and McCracken *et al.*, 1997). In addition, GT is also recruited to the CTD by SPT5, a component of the early elongation complex, which positively affects capping activity (Wen and Shatkin, 1999). Therefore, capping enzymes are recruited to the transcription complex following promoter clearance by the Ser 5 phosphorylated form of the CTD and the DSIF subunit SPT5 (Shilatifard *et al.*, 2003; Sims *et al.*, 2004 and Zorio and Bentley, 2004). As such, the capping process coincides with the early pause in Pol II elongation. Following completion of the 5' cap on the nascent RNA chain, the elongation repressor NELF is released from the paused transcriptional complex (Bentley, 2005). Furthermore, a phosphatase, possibly Fcp1, is responsible for the removal of Ser 5 phosphates from the CTD, which correlates with dissociation of the capping enzymes from the CTD (Bentley, 2002).

Addition of a 5' cap to the pre-mRNA transcripts may be an important checkpoint prior to productive elongation, as an intact 5' cap is necessary to protect the pre-mRNA from 5' to 3' exonucleases, as well as for slicing and polyadenylation events to be carried out (Neugebauer, 2002; Orphanides and Reinberg, 2002 and Proudfoot *et al.*, 2002). Cap binding complex (CBC), composed of cap binding protein 20 (CBP20) and 80 (CBP80), interacts co-transcriptionally with the cap structure in the nucleus (Proudfoot *et al.*, 2002). CBC is utilized in removal of the earliest pre-mRNA intron, stimulates cleavage of the 3'-end of the nascent RNA transcript, functions in mRNA export and continues to interact with the 5' cap of mature mRNAs until it is replaced by

the translation initiation factor eIF-4E in the cytoplasm (Neugebauer, 2002; Orphanides and Reinberg, 2002; Proudfoot *et al.*, 2002; Bentley, 2005 and Cheng *et al.*, 2006). Therefore, the co-transcriptional 5' cap processing reactions, mediated by the Pol II CTD, are critical for mRNA stability, additional processing events, mRNA export and prospective translation.

#### **1.4.1.2. Intron Splicing During Productive Elongation**

The production of functional and mature mammalian mRNAs requires that large noncoding intronic sequences, surrounding small coding exons, are excised from pre-mRNAs and that exons are joined together. However, intron removal, or splicing is not necessarily straight forward, as alternative splicing incorporates or omits different exons into mature mRNA products in as many as 75% of human genes to facilitate differential protein expression (Lynch, 2006). Splicing is carried out by a multi-protein complex called the spliceosome (Neugebauer, 2002; Proudfoot *et al.*, 2002 and Zorio and Bentley, 2004). The spliceosome harbors five small nuclear ribonucleoproteins (snRNPs): U1 snRNP, U2 snRNP, U4 snRNP, U5 snRNP and U6 snRNP, as well as numerous associated proteins (Neugebauer, 2002; Proudfoot *et al.* 2002 and Zorio and Bentley, 2004). Various components of the spliceosome recognize consensus sequences at the 5' splice site, the branchpoint located 100 bp upstream of the 3' intron-exon border, and the 3' intron-exon boundary of pre-mRNAs (Proudfoot *et al.*, 2002). Following identification of the 5' splice site by U1 snRNP, the 3' intron-exon boundary by the U2 snRNP auxiliary factor (U2AF), the branchpoint by U2 snRNP, and subsequent spliceosome assembly, intron splicing proceeds by two *trans*-esterification reactions (Neugebauer, 2002). First, a nucleophilic attack is carried out by the hydroxyl group from a conserved branchpoint adenosine nucleotide on the 5' exon-intron junction, leaving an open 5' boundary with a 3'OH group (Proudfoot *et al.*, 2002). Consequently, the free 3'OH of the 5' splice site behaves as a nucleophile by attacking the 3' intron-exon junction to release the intron (Proudfoot *et al.*, 2002). CBC may facilitate splicing of the first intron by stimulating interactions between the 5' recognition splice site and various components of the spliceosome (Proudfoot *et al.*, 2002).

It has been recognized for some time that the CTD of Pol II enhances intron splicing of the nascent RNA transcripts and slicing proceeds co-transcriptionally. However, the identification of slicing factors associated with the Pol II CTD or the transcriptional machinery in mammals has lagged considerably (McCracken *et al.*, 1997b and Fong and Bentley, 2001). Recently, four pre-mRNA splicing factors have been reported to associate with the transcriptional unit of the intron-containing human FOS gene (Listerman *et al.*, 2006). The U2AF 65kDa subunit (U2AF65), U1 snRNP, U5 snRNP and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) accumulate on the gene following upregulation of transcription, and correlate with co-transcriptional splicing of the pre-mRNA (Listerman *et al.*, 2006). Furthermore, U2AF65 interacts with the phosphorylated form of the Pol II CTD in a manner dependent on nascent RNA transcript association (Listerman *et al.*, 2006). Therefore, at least a percentage of intron splicing occurs during Pol II driven productive elongation. Interestingly, effective co-transcriptional splicing of the final intron and the presence of U2AF65 is strongly interconnected with 3' polyadenylation processing (Zhao *et al.*, 1999). In addition, pre-mRNA splicing enhances mRNA export to the cytoplasm (Orphanides and Reinberg, 2002).

#### **1.4.1.3. 3' Polyadenylation of Precursor-mRNA and Transcript Termination**

Polyadenylation of the pre-mRNA is carried out by the endonucleolytic cleavage of the nascent RNA chain and the addition of approximately 200 adenylate nucleotides to the cleaved 3' end of the transcript (Shilatifard, 2003). Cleavage and polyadenylation reactions are largely defined by poly(A) signal sequences within the 3'-region of the nascent RNA transcript (Zhao *et al.*, 1999). In mammals, the poly(A) signal is characterized by three RNA sequences: an upstream AAUAAA motif, a poly(A) site or cleavage site, and a downstream element (DSE) (Zhao *et al.*, 1999 and Zarudnaya *et al.*, 2003) (Figure 1.3.). The highly conserved AAUAAA, or alternatively AUUAAA, hexamer motif is located within 11 to 23 nucleotides upstream of the cleavage / poly(A) site and is necessary for pre-mRNA cleavage and polyadenylation (Chen *et al.*, 1995). The DSE is comprised of less well conserved stretches of U- or GU-rich nucleotide tracts and typically resides 10 to 30 nucleotides downstream of the poly(A) site (Chen *et al.*, 1995 and Zarudnaya *et al.*, 2003). Collaboratively, the AAUAAA hexamer and the

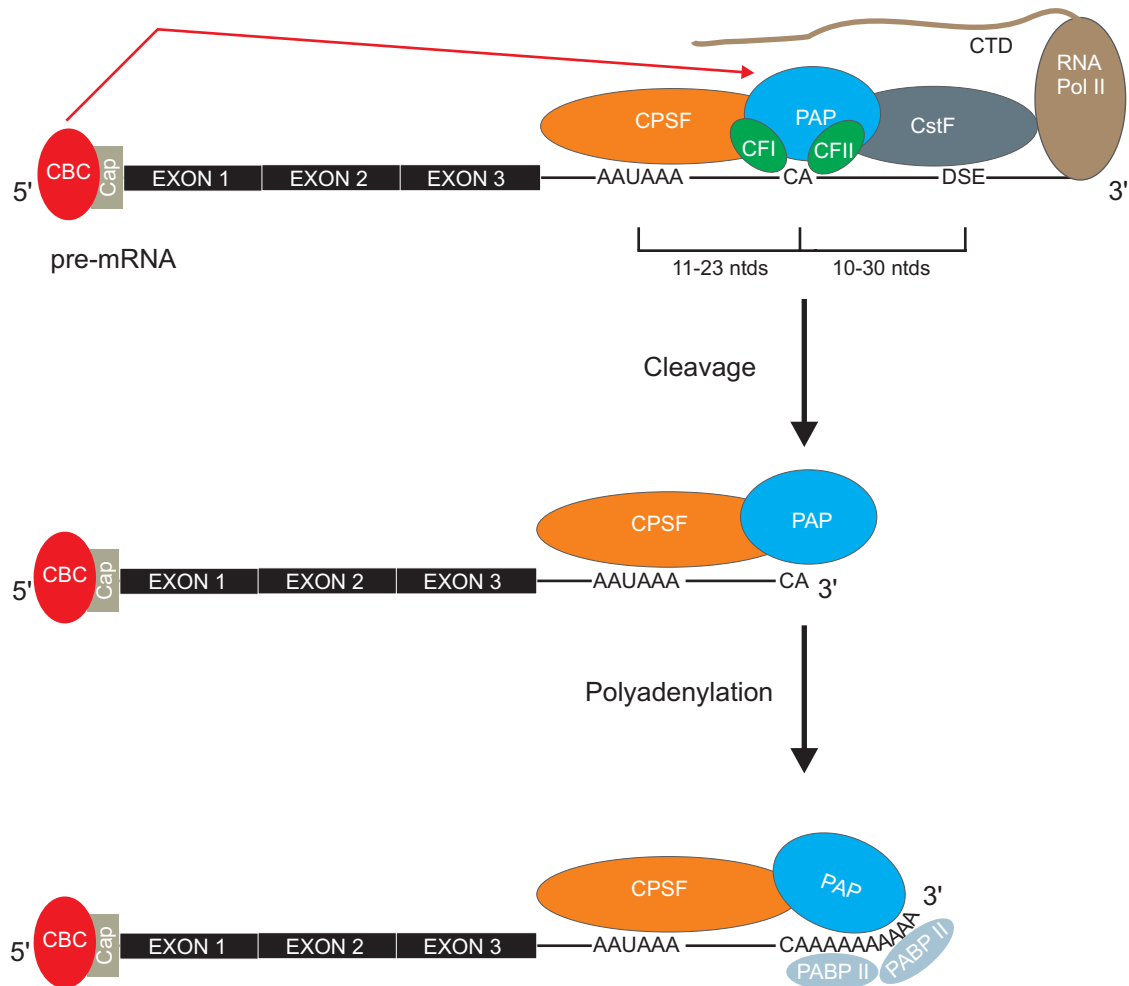


Figure 1.3. Polyadenylation of the pre-mRNA. Polyadenylation requires two distinct reactions. The endonucleolytic cleavage reaction is stimulated by CPSF, CstF, PAP, CFI and CFII. The polyadenylation reaction of the pre-mRNA 3'-end is mediated by CPSF, PAP and PABP II. The poly(A) signal sequences are denoted by the AAUAAA motif, CA cleavage site, and DSE. (Zhao *et al.*, 1999 and Proudfoot *et al.*, 2002)

DSE are thought to define the poly(A) / cleavage site, which is most often denoted by an adenosine nucleotide, and a penultimate cytosine (CA) (Chen *et al.*, 1995; Wahle and Ruegsegger, 1999 and Zhao *et al.*, 1999). Five multisubunit proteins are required for pre-mRNA endonucleolytic cleavage: cleavage and polyadenylation specificity factor (CPSF), cleavage stimulatory factor (CstF), cleavage factor I (CFI), cleavage factor II (CFII) and poly(A) polymerase (PAP) (Wahle and Ruegsegger, 1999; Zhao *et al.*, 1999 and Proudfoot *et al.*, 2002). Following cleavage, polyadenylation is carried out by CPSF, PAP and poly(A) binding protein II (PABP II) (Wahle and Ruegsegger, 1999; Zhao *et al.*, 1999 and Proudfoot *et al.*, 2002).

Cleavage of the pre-mRNA is initiated by CPSF and CstF which recognize and interact with the AAUAAA motif and DSE, respectively (Wahle and Ruegsegger, 1999 and Proudfoot *et al.*, 2002). Furthermore, interactions between CPSF and CstF stabilize the core of the cleavage complex (Zhao *et al.* 1999). Subsequent recruitment of PAP to CPSF, in addition to the association of CFI and CFII, completes the active complex and dictates cleavage of the nascent transcript (Figure 1.3.) (Proudfoot *et al.*, 2002). In addition, the CBC at the 5' end of the pre-mRNA stimulates the cleavage reaction. Polyadenylation proceeds immediately following cleavage by the concerted action of CPSF-bound PAP, and PABP II further stabilizes the complex after the addition of ten nucleotides to the mRNA tail (Figure 1.3.) (Wahle and Ruegsegger, 1999). Polyadenylation of the mRNA transcript is important for excision of the very last pre-mRNA intron, as U2AF65 interacts with PAP (Neugebauer, 2002). Furthermore, the poly(A) tail protects the 3'-end of the mRNA from rapid 3' to 5' exoribonuclease degradation.

In a comparable fashion to capping enzymes and splicing factors, proteins involved in 3'-end formation of the pre-mRNA also interact with the Pol II CTD. Both CPSF and CstF bind to the hypophosphorylated and hyperphosphorylated forms of the CTD *in vitro* (McCracken *et al.*, 1997b). Additionally, 3'-end processing factors have also been reported to associate with components of the transcriptional apparatus, such as TFIID, which directly interacts with CPSF, and the transcriptional coactivator positive cofactor 4, which binds CstF (Dantonel *et al.*, 1997 and Calvo and Manely, 2001). Interestingly, CPSF appears to interact with TFIID during formation of the PIC, but

later associates with the CTD during transcript elongation and, subsequently, the poly(A) signal of the pre-mRNA during 3'-end processing. (Dantonel *et al.*, 1997). Therefore, CPSF, and possibly CstF, are likely recruited to the PIC and transferred to the Pol II CTD upon TFIIH mediated phosphorylation of Ser 5 and promoter clearance (Calvo and Manley, 2003). Furthermore, CPSF and CstF are carried by the CTD throughout elongation, but dissociate from the Pol II CTD to interact with the nascent RNA hexamer AAUAAA motif and DSE, respectively, to initiate co-transcriptional endonucleolytic cleavage and polyadenylation of the 3' pre-mRNA (Calvo and Manley, 2003).

Polyadenylation is also intimately linked to Pol II transcript termination. Although precise consensus terminator sequences have yet to be identified, the poly(A) signal is necessary to halt RNA synthesis and stimulate Pol II release from the DNA template to allow recycling back to the PIC (Proudfoot, 2004; Zorio and Bentley, 2004; Buratowski, 2005 and Gromak *et al.*, 2006). Termination of transcription often occurs many bp downstream of the poly(A) addition site; pause sequences downstream of the poly(A) signal also play a role in termination (Yonaha and Proudfoot, 1999 and Gromak *et al.*, 2006). There are currently two models that explain the connection between polyadenylation and termination of transcription: the 'anti-terminator' model and the 'torpedo' model (Proudfoot *et al.*, 2002 and Buratowski, 2005). In the 'anti-terminator' model, synthesis of the pre-mRNA poly(A) signal leads to a change in Pol II-associated factors, such as the dissociation of positive elongation factors or the recruitment of negative elongation factors, which ultimately suppress the processivity of Pol II and consequently lead to Pol II release (Proudfoot *et al.*, 2002 and Buratowski, 2005). Conversely, the 'torpedo' model stipulates that upon pre-mRNA cleavage a new uncapped 5'-RNA end is generated from the surplus transcript length that is gradually degraded by the 5' to 3' exoribonuclease Xrn2, and eventually promotes dissociation of paused Pol II (Proudfoot *et al.*, 2002 and Buratowski, 2005). Therefore, 3'-end formation represents another example of the interrelated nature of transcription and pre-mRNA processing events.



#### **1.4.2. *cis*-Acting mRNA Stability Determinants**

The regulation of mRNA stability is another important area of gene expression control. Numerous factors influence mRNA rates of decay, including *cis*-acting stability determinants, secondary structure, the cellular setting of the mRNA, and the rate of translation (Ross, 1995). The following section will focus on examples of *cis*-acting mRNA stability determinants and the *trans*-acting mRNA binding factors that associate with mRNAs to regulate stability.

##### **1.4.2.1. The mRNA Cap and 5' Untranslated Region**

At the very 5'-end of the mRNA moiety, the 7-methyl-guanosine cap is an important stability determinant which enhances mRNA stability a minimum of fourfold *in vitro* (Ross, 1995). The cap structure fundamentally protects mRNAs from 5' to 3' exonuclease activity, as well as interacts with the translation initiation factor eIF-4E to promote further stabilization of the mRNA and attachment to the 43S ribosome for translation (Wilusz *et al.*, 2001 and Newbury, 2006). The 5' untranslated region (UTR) may influence mRNA half-life by the presence of hairpin loop structures, alteration of 5' UTR length or by the presence of stability determinants (Table 1.2.) (Guhaniyogi and Brewer, 2001). For example, the iron responsive element (IRE) stem-loop structure, present in the 5' UTR of human ferritin mRNA, may be bound by iron regulatory proteins (IRPs) during conditions of low iron (Ross, 1995 and Theil, 2000). Binding of the IRPs to the stem-loop within the 5' UTR sterically hinders the ferritin mRNA 5' cap from interacting with the 43S ribosome and thus has an inhibitory effect on translation, which ultimately precludes ferritin sequestration of iron (Ross, 1995 and Theil, 2000). A further example specifies how lengthening the 5' UTR can enhance mRNA stability, as the introduction of immunoglobulin 5' UTR sequence into c-myc, by reciprocal translocation, produces a significantly more stable mRNA product than wild-type c-myc mRNA (Ross, 1995). In addition, the 5' UTR may also contain sequence determinants that influence mRNA stability. The human interleukin-2 mRNA is stabilized during T-cell activation by the interaction of the RNA binding proteins nucleolin and Y-box 1 with a 5' UTR *cis* Jun N-terminal kinase response element (Chen *et al.*, 2000).




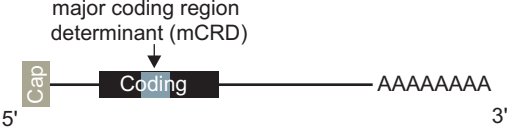
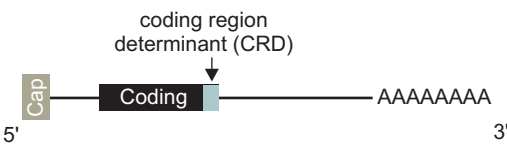

Location	Determinant	mRNA	Binding Proteins
5'UTR	Iron responsive element (IRE) 	Ferritin	Iron response protein
	Immunoglobulin 5'UTR 	c-myc	
	N-terminal jun kinase response element 	interleukin-2	nucleolin Y-Box 1
Coding Region	major coding region determinant (mCRD) 	c-fos	UNR
	coding region determinant (CRD) 	c-myc	CRD-binding protein
	M-R-E-I 	$\beta$ -tubulin	

Table 1.2. mRNA *cis*-acting stability determinants of the 5' UTR and coding region. Examples of *cis*-stability determinants and their *trans*-binding proteins are highlighted for different mRNAs. The Cap structure represents the 5'-end of the mRNA, followed by the 5' UTR (straight line), coding region (solid black box), the 3' UTR and 3'-end poly(A) tail. Sequence determinants are denoted by arrows. (Ross, 1995 and Guhaniyogi and Brewer, 2001)

#### **1.4.2.2. Coding region**

The coding regions of c-fos, c-myc and  $\beta$ -tubulin have been identified to carry sequence instability determinants (Ross, 1995 and Guhaniyogi and Brewer, 2001). c-fos mRNA contains a major coding region determinant (mCRD), which is 320 nucleotides in length, centrally located in the coding region of the mRNA and responsible for encoding the essential basic leucine zipper domain of c-fos (Table 1.2.) (Ross, 1995 and Guhaniyogi and Brewer, 2001). The mCRD associates with five proteins including the RNA binding protein, upstream of N-Ras (UNR), the cytoplasmic PABP, PABP-interacting protein, hnRNP D and hnRNP Q (Chang *et al.*, 2004). mCRD bound by UNR mediates rapid destabilization of c-fos mRNA upon recruitment of a multiprotein deadenylation / decay complex to the instability determinant, in a translation dependent manner (Chang *et al.*, 2004). The instability or coding region determinant (CRD) of c-myc resides in the most C-terminal 249 coding nucleotides of the mRNA, and interacts with a CRD-binding protein which plays a protective role against endoribonucleolytic cleavage during translational pauses (Herrick and Ross, 1994 and Lemm and Ross, 2002). However, dissociation of CRD-BP from the CRD at the ribosome appears to open up the c-myc mRNA for endonucleolytic attack, by RNase1- like endoribonuclease, during pauses in translation (Lemm and Ross, 2002 and Tafech *et al.*, 2007). Microtubules are comprised of heterodimer  $\alpha$ -tubulin and  $\beta$ -tubulin subunits.  $\beta$ -tubulin mRNA stability, which is autoregulated by the intracellular concentration of the unassembled tubulin subunit pool, is mediated by the first four N-terminal amino acids of  $\beta$ -tubulin, M-R-E-I (Yen, *et al.*, 1988 and Bachurski *et al.*, 1994). These amino acids likely interact with a peptide binding factor, and trigger degradation of the remaining mRNA sequence as translation proceeds (Yen, *et al.*, 1988 and Bachurski *et al.*, 1994).

#### **1.4.2.3. The Poly(A) Tail and 3'Untranslated Region**

The 3' poly(A) tail of the mRNA functions in a manner similar to the 5' cap, given that progressive shortening of the poly(A) tail to approximately 30 to 65 nucleotides stimulates rapid and irreversible mRNA decay (Ford *et al.*, 1999). Fundamentally, the poly (A) tail is protected, by bound cytoplasmic poly(A) binding proteins (PABPs), from the action of 3' to 5' exonucleases (Ford *et al.*, 1999). In addition to the poly(A) tail, the mRNA 3' UTR has been documented to possess a

wealth of *cis*-acting stability elements. Examples of 3' UTR determinants that influence mRNA stability include the  $\alpha$ -globin C-rich element, transferrin receptor IREs, insulin-like growth factor II (IGF II) long range stem loop, histone stem loop and AU-rich elements (Table 1.3.). The C-rich element associated with the 3' UTR of  $\alpha$ -globin facilitates formation of an  $\alpha$ -globin stabilizing  $\alpha$ -complex (Waggoner and Liebhaber, 2003). The  $\alpha$ -complex is composed of  $\alpha$ -globin poly(C) binding protein 1 ( $\alpha$ CP1) and 2 ( $\alpha$ CP2). This complex shields the  $\alpha$ -globin mRNA from endoribonucleolytic cleavage and prevents deadenylation by interacting with PABPs bound to the poly (A) tail (Waggoner and Liebhaber, 2003). The 3' UTR of the transferrin receptor contains five stem-loop IREs that interact with IRPs when intracellular iron levels are low (Theil, 2000). However, in contrast to ferritin mRNA, IRP binding to transferrin receptor IREs obstructs endonuclease access to the mRNA and, therefore, enhances transferrin receptor mRNA stability and stimulates iron uptake (Guahaniyogi and Brewer, 2001 and Theil, 2001). The mRNA stability of IGF-II is regulated by a long-range stem-loop determinant in the 3' UTR that is susceptible to endoribonucleolytic cleavage (Ross, 1995). Furthermore, replication dependent histone mRNAs lack a poly(A) tail, but possess a 3' terminal stem loop structure that mediates their mRNA decay upon interaction with a stem-loop binding protein (Guahaniyogi and Brewer, 2001).

Undoubtedly, the preeminent example of a 3' UTR determinant is a sequence element high in adenosine and uridine nucleotides, known as an AU-rich element (ARE). AREs have been estimated to reside in 5-8% of human mRNAs, including those of cytokines, transcription factors, proto-oncogenes, and growth regulatory proteins (Wilusz *et al.*, 2001; Barreau *et al.*, 2005 and Bakheet *et al.*, 2006). Interestingly, AREs are powerful instability elements that vary from 50-150 nucleotides and are, therefore, usually found in the 3' UTRs of short lived mRNAs (Chen and Shyu, 1995). AREs can be divided into three classes (Chen and Shyu, 1995 and Barreau *et al.*, 2005). Class I AREs have 1-5 copies of the AUUUA pentamer motif within a U-rich environment and are present in the c-fos, c-myc and p21WAF1 mRNAs (el-Deiry *et al.*, 1993; Chen and Shyu, 1995 and Barreau *et al.*, 2005). Granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF- $\alpha$ ) mRNAs contain Class II AREs, which possess at least two overlapping UUAUUUA(U/A)(U/A) nonamers in a

Location	Determinant	mRNA	Binding Proteins
3'UTR	<p>C-rich element</p>	$\alpha$ -globin	$\alpha$ CP1, $\alpha$ CP2 ( $\alpha$ -complex)
	<p>IREs</p>	transferrin receptor	IRPs
	<p>long range stem loop</p>	IGF-II	
	<p>3' terminal stem loop</p>	histone	stem loop binding protein
	<p>Class I ARE (AUUUA)<sub>1-5</sub></p>	c-fos c-myc p21WAF1	AUF1 HuR
	<p>Class II ARE UUAUUUA(U/A)(U/A)</p>	GM-CSF TNF- $\alpha$	AUF1 HuR TTP
	<p>Class III ARE U-rich</p>	c-jun	

Table 1.3. *Cis*-acting stability determinants of the mRNA 3' UTR. Examples of *cis*-stability determinants and their *trans*-binding proteins are highlighted for different mRNAs. The 3' UTR is represented by the solid line preceding the poly(A) tail. Sequence determinants are denoted by arrows. (Ross, 1995 and Guhaniyogi and Brewer, 2001)

U-rich context (Chen and Shyu, 1995 and Barreau *et al.*, 2005). Lastly, the Class III AREs are U-rich but do not have an AUUUA motif and are found in mRNAs including the c-jun mRNA (Chen and Shyu, 1995 and Barreau *et al.*, 2005). The potent destabilizing nature of AREs was initially observed upon insertion of an ARE from GM-CSF into the 3'UTR of a previously stable  $\beta$ -globin gene (Chen and Shyu, 1995 and Ross, 1995). This destabilizing phenomenon can be explained by the ability of AREs to mediate rapid deadenylation, and the subsequent 3' to 5' or 5' to 3' exonuclease degradation of mRNAs (Chen and Shyu, 1995 and Stoecklin *et al.*, 2006).

A number of *trans*-associated factors have been identified to interact with AREs, however, only AU-rich binding factor 1 (AUF1), members of the embryonic lethal abnormal visual (ELAV) family and the tristetraprolin (TTP) family impart either positive or negative effects on mRNA stability in response to cellular stimuli. The first ARE binding protein to be characterized was the nucleo-cytoplasmic shuttling protein hnRNP D, otherwise known as AUF1 (Barreau *et al.*, 2005). *In vitro* RNA binding studies have identified that AUF1 interacts with AREs from multiple mRNAs, including c-fos, c-myc, p21WAF1, GM-CSF and TNF- $\alpha$  mRNAs (Barreau *et al.*, 2005). However, the effect that AUF1 elicits on mRNA stabilization remains controversial, as AUF1 has been reported to increase the decay rate of c-fos, c-myc, p21WAF1 and GM-CSF ARE-containing mRNAs, but has also been proven to enhance stabilization of c-fos, c-myc, GM-CSF and TNF- $\alpha$  ARE-associated mRNAs in different cell lines (Loflin *et al.*, 1999; Xu *et al.*, 2001 and Barreau *et al.*, 2005).

Members of the ELAV family represent a group of evolutionary conserved ARE binding proteins, and are represented by the ubiquitously expressed HuR and neuronal HuC, HuD and Hel-N1 members (Antic and Keene, 1997). HuR is the most thoroughly characterized of the human ELAV proteins. It is primarily situated in the nucleus, although it may shuttle between the nucleus and the cytoplasm, and has been reported to target a number of ARE-containing mRNAs, such as c-fos, c-myc, p21WAF1, GM-CSF and TNF- $\alpha$  (Brennan and Steitz, 2001 and Barreau *et al.*, 2005). HuD association with the 3' UTRs of these mRNAs occurs through a 17 to 20 nucleotide U-rich motif predicted to display a hairpin secondary structure (Silanes *et al.*, 2004). Unlike AUF1, a more clearly defined role exists for HuR, which has extensively been reported to

stabilize levels of mRNAs containing Class I, Class II and Class III AREs (Peng *et al.*, 1998 and Barreau *et al.*, 2005). Importantly, HuR does not appear to inhibit mRNA decay by preventing mRNA deadenylation, but may retard mRNA decay by stabilizing deadenylated intermediates and inhibiting exonuclease access (Peng *et al.*, 1998 and Ford *et al.*, 1999). In addition, HuD, HuC and Hel-N1 have also been linked to enhanced stabilization of ARE-containing mRNAs (Barreau *et al.*, 2005).

TTP is the newest ARE binding protein to be investigated. TTP is a CCCH tandem zinc finger protein that interacts with the consensus nonamer UUAUUUAUU of ARE associated mRNAs, and has notably been documented to trigger the decay of mRNAs possessing Class II AREs, such as GM-CSF and TNF- $\alpha$  (Lai *et al.*, 2006). The mechanism by which TTP triggers destabilization of mRNA is unclear, however 5' to 3' and 3' to 5' exonuclease components have been localized to TTP bound ARE sequences, suggesting that TTP may stimulate decay through the recruitment of exonuclease factors to the mRNA (Hau *et al.*, 2006). Thus, given the sheer number of identified mRNA *cis*-acting stability and instability determinants, as well as the broad array of *trans*-acting factors, it is easy to conclude that mRNA stabilization is an important juncture in the regulation of gene expression.

## **1.5. c-Src and the c-Src Family Kinase Members**

### **1.5.1. Overview of the Rous Sarcoma Virus, v-Src and the c-Src Proto-oncogene**

In 1911, Peyton Rous made a revolutionary contribution to the field of tumor biology when he observed that a cell-free filterable agent derived from a chicken sarcoma could induce the formation of a solid tumor in a new host (Rous, 1911). However, his work met with a considerable level of skepticism, due to the belief that an infectious agent could not be the cause of a malignancy; it was not until years later that the Rous Sarcoma Virus (RSV) was finally accepted as the agent responsible for this tumor production. Subsequent work demonstrated that RSV not only induced transformation, but that the resulting tumor cells had an enhanced survival rate and both produced and released the virus, thereby suggesting that RSV played a fundamental role in tumor propagation (Rubin, 1955). Furthermore, RSV was also shown to induce

morphological changes in tumor cells (Temin and Rubin, 1958). Over a decade later, the theory that a gene encoded in the RNA genome of RSV may be responsible for its transformation potential was spurred by the observations that mutant strains of RSV displayed transformation defects but were still competent for replication (Golde, 1970 and Martin, 1970). In addition, the genomes of replication and transformation proficient RSV strains were reported to be larger in size than the genomes of mutant RSV strains defective in transformation (Duesberg and Vogt, 1970 and Martin, 2001). This suggested that the ribonucleotide sequence missing from the non-transformed RSV strains likely harbored the transforming gene. The retroviral oncogene accountable for the transforming potential of RSV, viral-Src (v-Src), was later sequenced (Czernilofsky, *et al.*, 1980 and Czernilofsky, *et al.*, 1983). Interestingly, a normal cellular counterpart of v-Src, named cellular-Src (c-Src), was detected upon hybridization of a complementary DNA v-Src probe to normal vertebrate DNA (Stehelin *et al.*, 1976). c-Src was denoted the first proto-oncogene, given its nature as a cellular precursor to the retroviral transforming gene, although it displayed inferior transforming activity toward chicken embryo fibroblast compared to its viral homologue (Iba *et al.*, 1984 and Martin, 2001). However, a role for c-Src in cancer has been extensively documented, as aberrant regulation leading to overexpression and/or activation of c-Src have been linked to events such as cellular transformation, tumorigenesis and metastasis (Summy and Gallick, 2003).

### **1.5.2. c-Src Family Kinase Protein Structure and Regulation**

The gene products of the v-Src and c-Src genes encode highly similar 60 kDa non-receptor tyrosine kinases. v-Src, however, possesses 18 point mutations and is truncated by the replacement of 19 amino acids with 12 different amino acids at the C-terminal end of the protein (Takeya and Hanafusa, 1983). c-Src is the archetype for a group of related c-Src family non-receptor tyrosine kinase (SFK) family members including: c-Yes, Fyn, Lyn, Lck, Hck, Fgr and Blk (Thomas and Brugge, 1997). The protein structures of c-Src and the SFK members range in size from 52-62 kDa and are similarly comprised of six functional domains, the N-terminal Src homology 4 (SH4) domain, the unique domain, the SH3 domain, the SH2 domain, the kinase or SH1



domain, and the C-terminal regulatory domain (Figure 1.4.) (Thomas and Brugge, 1997 and Boggon and Eck, 2004). The SH4 domain is 14 amino acids in length and fosters the co-translational covalent linkage of myristic acid and, occasionally, palmitic acid at the very N-terminal of the SFK proteins (Biscardi *et al.*, 1999 and Dehm and Bonham, 2004a). These linkages tether the SFKs to the plasma membrane and facilitate interactions between signaling molecules, such as membrane bound receptor tyrosine kinases and integrin associated focal adhesions. The unique domain, of 40-70 amino acids, is weakly conserved among the SFKs and may be involved in mediating specific protein-protein interactions with individual family members, although the function of this domain is poorly understood (Brown and Cooper, 1996; Biscardi *et al.*, 1999 and Boggon and Eck, 2004). Further, protein interactions with SFKs occur through the approximately 50 amino acid SH3 domain and the roughly 100 amino acid SH2 domain, which bind to proline-rich consensus sequences (P-X-X-P) and phosphotyrosine (pY) residues, respectively (Thomas and Brugge, 1997 and Alvarez *et al.*, 2006). The SH2 domain has two binding pockets that prefer to interact with the pY-E-E-I amino acid sequence of proteins, where one pocket interacts with the phosphotyrosine (pY) residue and the other with the third amino acid residue downstream of the phosphotyrosine residue (Roskoski, 2004 and Thomas and Brugge, 1999). The SH1 domain is a highly conserved, 250 amino acid tyrosine kinase catalytic domain that harbors a tyrosine residue within the activation loop that is critical for complete kinase activation of the SFK members (Brown and Cooper, 1996 and Boggon and Eck, 2004). The activating residue in the SH1 domain of c-Src resides at Y416 in chickens and Y419 in humans (Bjorge *et al.*, 2000a). Finally, a 15-17 amino acid C-terminal regulatory domain holds an essential autoinhibitory phosphorylation site, which negatively regulates SFKs (Boggon and Eck, 2004). The c-Src autoinhibitory residue is Y527 in chickens and Y530 in humans (Brown and Cooper, 1996). v-Src lacks this autoinhibitory tyrosine residue (Takeya and Hanafusa, 1983).

Regulation of SFK activity is maintained by a dynamic interplay of phosphorylation and dephosphorylation events in coordination with intramolecular protein interactions (Frame, 2002). Inactivation of c-Src occurs following carboxy-terminal Src kinase (Csk) phosphorylation of the autoinhibitory Y527 residue,

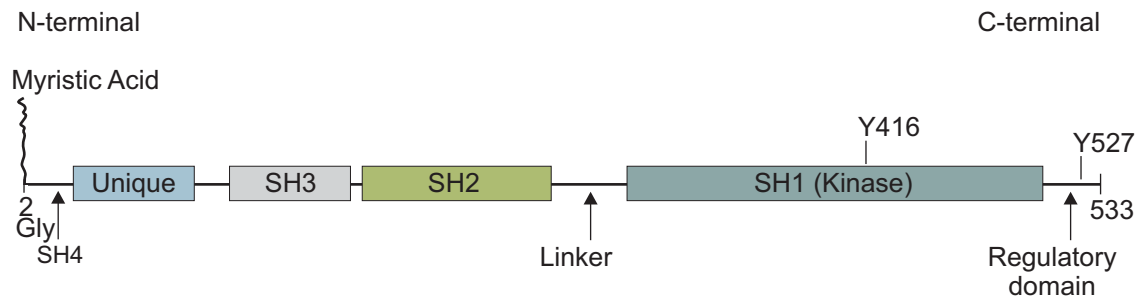


Figure 1.4. The protein structure of chicken c-Src. The SH4 myristolation domain, Unique domain, SH3 domain, SH2 domain, Linker, SH1 catalytic kinase domain and regulatory domain are shown from the N- to C-terminal of the protein. The important regulatory tyrosine (Y) residues are highlighted.

prompting c-Src to bind its own SH2 domain, resulting in a “closed” conformation (Okada and Nakagawa, 1989). In addition, the SH3 domain contributes to the inactive kinase state by interacting with a linker region between the SH2 and the kinase domain (Xu *et al.*, 1997). An open and active c-Src kinase configuration, on the other hand, can be stimulated by dephosphorylation of the Y527 by phosphatases, such as protein tyrosine phosphatase 1B (Bjorge *et al.*, 2000b). A decrease in Csk activity also stimulates c-Src activation while direct phosphorylation of Y416 positively activates the c-Src kinase domain and encourages substrate interactions (Bjorge *et al.*, 2000a and Cam *et al.*, 2001). Also, higher affinity binding proteins, such as platelet-derived growth factor receptor (PDGFR) and focal adhesion kinase (FAK), may compete for c-Src SH2 and SH3 domains to disrupt intramolecular interactions and activate c-Src (Bjorge *et al.*, 2000a; Frame, 2002 and Yeatman, 2004). Moreover, mutation of Y527 or deletion of the c-Src C-terminal domain, as observed in v-Src, leads to constitutive activation of c-Src and the enhancement of cellular transformation (Cartwright *et al.*, 1987).

### **1.5.3. Signaling Events Mediated by the c-Src Family Kinase Members**

Members of the family of c-Src kinases are differentially expressed across tissues. c-Src, Yes, and Fyn are expressed ubiquitously, while expression of the remaining SFKs Lck, Lyn, Hck, Blk and Fgr is primarily localized to hematopoietic cells (Thomas and Brugge, 1997). SFK members contribute to various signaling pathways, in different cellular contexts, through cell surface receptors including receptor protein tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), integrin receptors, cadherin cell to cell adhesion molecules, and antigen receptors (Thomas and Brugge, 1997 and Parsons and Parsons, 2004). For example, c-Src, Yes and Fyn are activated upon stimulation of many RTKs and have been shown to mediate signaling of PDGFR, EGFR, FGFR, insulin growth factor receptor 1 receptor (IGF1R) and colony stimulating growth factor 1 receptor (CSF1R), in addition to a number of other RTKs, to positively regulate mitogenic events (Frame, 2002 and Bromann *et al.*, 2004). While in exchange, SFKs have been reported to reciprocally transactivate RTKs, and regulate RTK turnover (Bromann *et al.*, 2004). Hormone stimulated GPCRs

enhance c-Src, Yes and Fyn recruitment and kinase activity, either by direct interaction with components of the GPCR or via crosstalk with the RTKs and focal adhesion complexes, to respectively control GPCR-containing vesicular trafficking, cellular proliferation and cytoskeleton rearrangements (Luttrell and Luttrell, 2004). Integrin receptors, bound by extracellular matrix proteins, facilitate the assembly of multi-protein focal adhesion complexes, which encompass numerous proteins including FAK, p130<sup>CAS</sup>, talin, paxillin, vinculin, and c-Src (Playford and Schaller, 2004). Activated c-Src, Yes and Fyn appear to stimulate focal adhesion disassembly, disrupt cell contact with the extracellular matrix and consequently enhance cellular motility (Playford and Schaller, 2004 and Yeatman, 2004). Furthermore, activated c-Src, in collaboration with FAK, has been implicated in the negation of cellular apoptosis resulting from cellular detachment from the extracellular matrix (Playford and Schaller, 2004). E-cadherin molecules from neighboring cells interact to form adherens junctions which activated c-Src has been documented to upset by inhibiting E-cadherin localization to the cell surface and promoting E-cadherin endocytosis (Yeatman, 2004). A deficit in E-cadherin levels has been shown to increase cell invasion, therefore, c-Src prevents cell adhesion and promotes both cellular motility and invasion (Yeatman, 2004). In a more tissue specific manner, Lck and Fyn are activated during thymocyte development and maintenance of peripheral T-cells by the T-cell antigen receptor (Palacios and Weiss, 2004). Similarly, Lyn, Fyn and Blk are activated by the B-cell antigen receptor to both positively and negatively regulate B-cell signaling events (Gauld and Cambier, 2004 and Geahlen *et al.*, 2004). Taken together, SFK members engage in a number of cellular signaling pathways associated with proliferation, differentiation, adhesion, migration, invasion, survival and development.

#### **1.5.4. c-Src Family Kinase Knockout Mice**

Given that the SFK members have been implicated in such a diverse array of signaling processes, individual SFK knockout mice were predicted to display major phenotypic abnormalities. However, it was surprising to observe that *src*<sup>-/-</sup> mice were viable and only developed a bone remodeling defect that resulted in bone buildup, called osteopetrosis (Soriano *et al.*, 1991). Hck and Fgr are two other SFKs, besides c-

Src, which are expressed at high levels in mouse osteoclasts (Lowell and Soriano, 1996). Interestingly, upon examination of *src*<sup>-/-</sup> mice, Hck protein levels were elevated in osteoclasts (Lowell and Soriano, 1996). Furthermore, the generation of double *src*<sup>-/-</sup> and *hck*<sup>-/-</sup> knockout mice produced a more severe osteopetrosis phenotype, suggesting that SFK members may compensate for one another in the absence of a family member (Lowell *et al.*, 1996). Of the remaining ubiquitously expressed SFK members, Yes and Fyn, *yes*<sup>-/-</sup> knockout mice have no visible defects, while *fyn*<sup>-/-</sup> mice exhibited deficiencies in hippocampal development, long term potentiation, memory and thymocyte signaling (Stein *et al.*, 1992 and Stein *et al.*, 1994). Double knockout mice of *src*<sup>-/-</sup> *yes*<sup>-/-</sup> and *src*<sup>-/-</sup> *fyn*<sup>-/-</sup>, however, died perinatally, whereas a portion of *yes*<sup>-/-</sup> *fyn*<sup>-/-</sup> mutant mice survived with renal disease (Stein *et al.*, 1994). In addition, other SFK knockout mice have been generated for SFK members expressed in a tissue specific manner. T-cells express high levels of Lck and Fyn. Mice possessing kinase dead Lck developed impairments in T-cell development and T-cell antigen receptor signaling, however, *fyn*<sup>-/-</sup> *lck*<sup>-/-</sup> double knockout mice were reported to display a significant 100-fold reduction in T-cells (Levin *et al.*, 1993 and Lowell and Soriano, 1996). Furthermore, high expression levels of Lyn, Fyn and Blk are present in B-cells. In accordance, *lyn*<sup>-/-</sup> knockout mice produced 50-65% less peripheral B-cells than wild type mice, while *blk*<sup>-/-</sup> mice have no abnormalities (Lowell and Soriano, 1996). Cumulatively, these observations suggest that there is significant functional redundancy between the SFK members.

#### **1.5.5. Role of the c-Src Family Kinase Members in Cancer**

Numerous groups have reported that aberrant regulation of SFK members is linked to cancer (Summy and Gallick, 2003). Of the SFK members, c-Src protein overexpression and/or enhanced kinase activity has been most thoroughly implicated in cellular transformation, tumor progression and metastasis (Biscardi *et al.*, 1999; Frame, 2002; Russello and Shore, 2003 and Summy and Gallick, 2003). Deregulation of c-Src, leading to high c-Src protein levels or elevated kinase levels, has been recurrently observed in colon and breast carcinomas, and less commonly detected in pancreatic, lung, brain, ovarian, bladder, gastric, head, neck and esophageal tumors (Summy and

Gallick, 2003). The relationship between c-Src and colorectal malignancies remains the most clearly defined example of c-Src involvement of tumorigenesis. Colon carcinoma cells and tissues prominently display up to a 40 fold increase in c-Src kinase activity when compared to normal adjacent colonic mucosa (Rosen *et al.*, 1986; Bolen *et al.*, 1987 and Cartwright *et al.*, 1989). Furthermore, enhanced c-Src kinase activity has been documented in early pre-malignant ulcerative colitis and colonic polyps (Cartwright *et al.*, 1990 and Cartwright *et al.*, 1994). As well, highly metastatic cells display constitutively elevated c-Src kinase activity above that in poorly metastatic cells in colon neoplasias (Han *et al.* 1996, and Mao *et al.*, 1997). In accordance, c-Src tyrosine kinase activity has been shown to increase with tumor progression, as pre-malignant polyps exhibit high c-Src kinase activity, which is elevated in primary colonic tumors, and greatest in hepatic metastases derived from colon carcinoma (Talamonti *et al.*, 1993 and Summy and Gallick, 2003). Recently, Gallick's group has reported that elevated c-Src kinase activity may serve as an independent indicator of poor clinical prognosis in colorectal carcinoma (Aligayer *et al.*, 2002). In addition, antisense studies targeting c-Src reduced tumor cell growth rate and tumorigenicity, indicating that c-Src may be an important therapeutic target in the treatment of carcinomas expressing high levels of c-Src (Staley *et al.*, 1997). In tumor cells, the rise in c-Src protein levels and kinase activity may be mediated by multiple mechanisms, such as the overexpression of RTKs, high FAK protein levels, or a decrease in Csk expression levels (Summy and Gallick, 2003). Moreover, activating c-Src mutations have been identified in a small percentage of highly advanced colon cancers, however, these results remain controversial (Irby *et al.*, 1999). Elevated c-Src expression and enhanced kinase activity have also, more recently, been attributed to increased transcriptional activation in a subset of colon cancer cell lines (Dehm *et al.*, 2001).

In addition to c-Src overexpression in cancer, deregulation of Yes, Lck, Lyn expression and kinase activity have also been implicated in tumor development, proliferation, motility, invasiveness and progression (Summy and Gallick, 2003). The kinase activity of Yes is commonly enhanced in premalignant colonic adenomas and is high in both primary colon carcinomas and liver metastatic lesions, compared to normal colonic mucosa (Park *et al.*, 1993; Pena *et al.*, 1995 and Han *et al.*, 1996). Furthermore,

augmented Yes protein kinase activity has been documented in breast, melanoma and small cell lung cancer cells (Loganzo *et al.*, 1993 and Krystal *et al.*, 1998). Lyn has been shown to be activated in T cell leukemia and lymphomas derived from B-cells, glioblastomas, and has also been reported to display significant levels of expression in colon and prostate cancer (Uchiumi *et al.*, 1992; Chen *et al.*, 1999; Bates *et al.*, 2001; Goldenberg-Furmanov *et al.*, 2004 and Stettner *et al.*, 2005). Lck overexpression and /or kinase activation have been observed in colon cancer, small cell lung cancer and lymphocytic leukemia cells (Veillette *et al.*, 1987; Abts *et al.*, 1991; Nakamura *et al.*, 1996 and Krystal *et al.*, 1998).

#### **1.5.6. SRC Gene Transcription**

The human SRC gene resides on chromosome 20q12.3 and is comprised of 15 exons (Figure 1.5.) (Anderson *et al.*, 1985; Tanaka *et al.*, 1985; Bonham and Fujita, 1993 and Bonham *et al.*, 2000). The first two exons, 1 $\alpha$  and 1A, are separated by approximately 1 kb and are associated with their own individual promoter, SRC1 $\alpha$  and SRC1A, respectively (Bonhman and Fujita, 1993 and Bonham *et al.*, 2000). Exons 1B and 1C encode for the 5' UTR of the c-Src mRNA while exons 2 through 12 code for the c-Src protein and the 3' noncoding region. Alternative usage of the SRC1A and SRC1 $\alpha$  promoters linked to their respective exons produce transcripts that commonly splice to exon 1B and, therefore, differ only in their extreme 5' UTR and so code for identical c-Src proteins (Figure 1.5.).

The SRC1A promoter exhibits characteristics consistent with a housekeeping gene promoter, such as the presence of a high GC content and multiple transcription start sites (Bonham and Fujita, 1993). Regulation of this promoter is mediated by the Sp family of zinc finger transcription factors and hnRNP K (Ritchie *et al.*, 2000 and Ritchie *et al.*, 2003). Two proximal promoter binding elements, GC1 and GA2, interact with the Sp1 and Sp3 members of the Sp family, while hnRNP K associates with three polypurine:polypyrimidine tracts, TC1, TC2 and TC3 (Figure 1.5.) (Ritchie *et al.*, 2000 and Ritchie *et al.*, 2003). Sp1 and Sp3 are ubiquitously expressed proteins that encode highly analogous structures and bind GC boxes with equivalent affinity, however, Sp1 and Sp3 differ in their DNA binding characteristics and regulatory abilities (Li *et al.*,

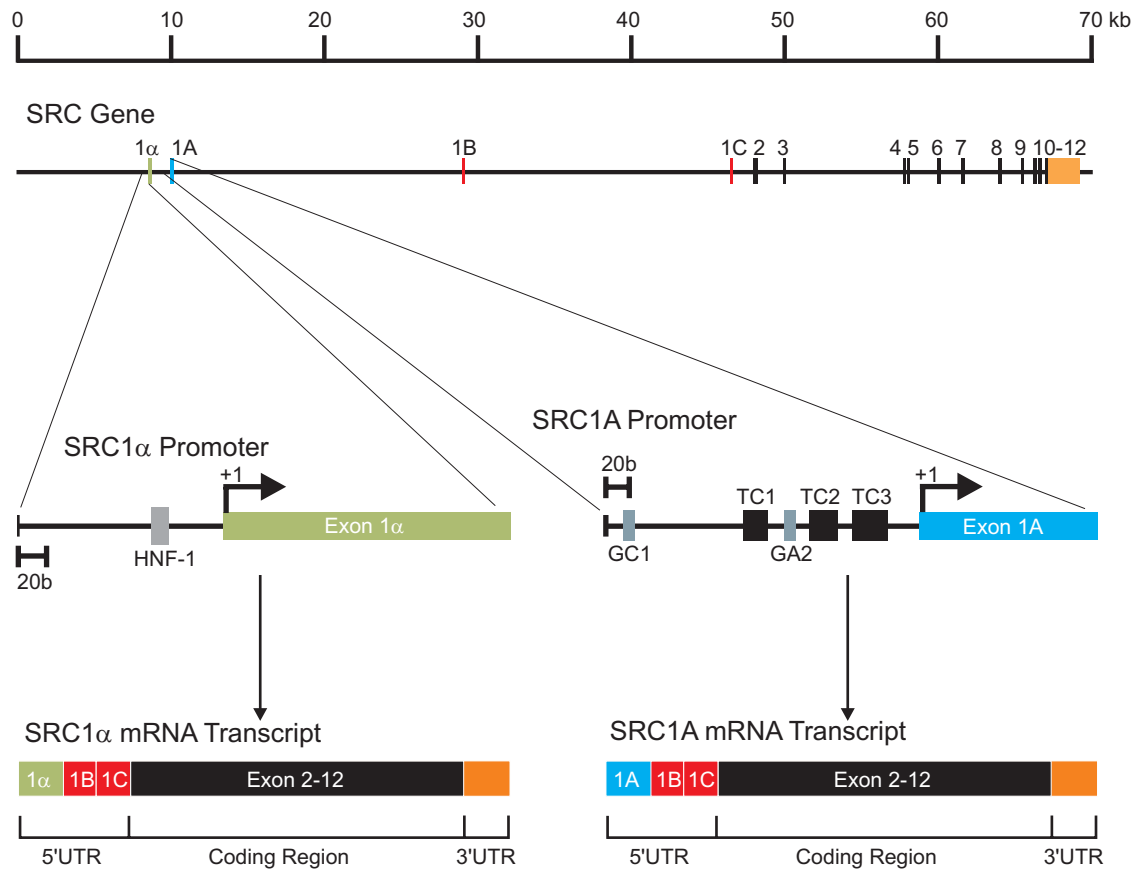


Figure 1.5. The human SRC gene. The organization of the SRC intron and exon (colored boxes) boundaries are denoted. The 5' non-coding SRC exons are 1α (green), 1A (blue), 1B, (red), and 1C (red). The SRC coding region and 3' UTR are encoded by exons 2 through 12 (black = coding region, orange = 3' UTR). The SRC1α and SRC1A promoters with their associated exons shown. The SRC1α promoter contains a *cis*-acting HNF-1 proximal promoter element. The SRC1A promoter is regulated by the GC1, GA2, TC1, TC2 and TC3 proximal promoter elements. The SRC promoters transcribe products that vary only in their extreme 5' end and commonly splice to Exon 1B. The arrows indicate the approximate transcription start sites. (Bonham and Fujita, 1997, Bonham *et al.*, 2000, Ritchie *et al.*, 2000 and Dehm *et al.*, 2004)



2004a). While Sp1 binds GC boxes as multimers and synergistically trans-activates promoters that possess multiple Sp binding sites, Sp3 binds DNA binding elements as a monomer and may act as either a transcriptional activator or repressor, based on the promoter context (Mastrangelo *et al.*, 1991; Su *et al.*, 1991; Yu *et al.*, 2003 and Li *et al.*, 2004a). In the case of the SRC1A promoter, both Sp1 and Sp3 activate SRC1A transcription through the GC1 and GA2 sites, although the sumoylated form of Sp3 may negatively regulate SRC1A transcription (Ritchie *et al.*, 2000 and Ellis *et al.*, 2006). Polypurine:polypyrimidine tracts have been reported to assume non-B-DNA structures and develop H-DNA conformations of triple- and single-stranded DNA (Mirkin and Frank-Kamenetskii, 1994). Interestingly, hnRNP K binds the TC1 and TC2 tracts in both double and single-stranded forms, and has single-stranded DNA binding ability toward the TC3 tract (Ritchie *et al.*, 2000). Cumulatively, Sp1/Sp3 interaction with the GC1 and GA2 sites and hnRNP K binding of TC1 and TC2 tracts cooperate to activate transcription and ensure ubiquitous expression of the SRC1A promoter (Ritchie *et al.*, 2000).

In contrast to the SRC1A promoter, the SRC1 $\alpha$  promoter is much more tissue specific; it is expressed primarily in stomach, pancreas, liver, kidney, prostate and gut tissues (Figure 1.5) (Bonham *et al.*, 2000). This promoter is controlled by a single hepatocyte nuclear factor-1 (HNF-1) proximal promoter DNA binding element and is regulated by the liver enriched transcription factor, HNF-1 $\alpha$  (Bonham *et al.*, 2000). Although the SRC1A and SRC1 $\alpha$  promoters differ vastly, both promoters lack a TATA-box proximal promoter element and have recently been documented to harbor potential Inr elements (Dehm *et al.*, 2004). Furthermore, these SRC1A and SRC1 $\alpha$  Inr elements directly interact with TAF1-TAF2 *in vitro* and have been demonstrated to possess TAF1 dependency in temperature sensitive (*ts*) *ts*BN462 cells (Dehm *et al.*, 2004). *ts*BN462 cells are derived from a baby hamster kidney cell line (BHK-21) that harbors a TAF1 mutation in the AT domain (G690A), which results in TAF1 AT inactivation, G1 cell cycle arrest and cell death at the nonpermissive temperature (Hayashida *et al.*, 1994 and Dunphy *et al.*, 2000).

### 1.5.7. The c-Src Family Kinase YES, LYN and LCK Gene Promoters

Relatively little is known about the YES and LYN promoters, aside from initial observations (Matsuzawa *et al.*, 1991 and Uchiumi *et al.*, 1992). The YES promoter, similar to the SRC1A promoter, is a ubiquitously expressed housekeeping-like promoter, lacking a TATA-box, possessing multiple transcription sites and a GC-rich sequence (Figure 1.6.) (Matsuzawa *et al.*, 1991). Moreover, five GC boxes have been identified in the YES promoter, four of which are immediately upstream of the major transcription start site, referred to as the GC-cluster (GC1-GC4), while the additional GC-box further upstream is called GC5 (Matsuzawa *et al.*, 1991). All five GC-boxes were shown to be important *cis*-acting regulatory sequences within the YES promoter, and Sp1 was documented to interact with GC5 as well as the four GC boxes in the cluster (Matsuzawa *et al.*, 1991). The LYN promoter is primarily expressed in B lymphocytes, and to a lesser degree in T cells (Uchiumi *et al.*, 1992). Interestingly, the LYN promoter also does not contain a TATA box, and harbors multiple transcription start sites as well as three GC box sequence motifs (Figure 1.6.) (Uchiumi *et al.*, 1992). In addition, an octamer transcription factor (OTF) consensus site in the LYN proximal promoter interacts with OTF-1 and likely facilitates LYN transcription (Uchiumi *et al.*, 1992). Surprisingly, the LCK gene has been quite well characterized and contains two distinct promoters spaced approximately 35 kb apart, LCK type I and LCK type II, which produce transcripts that diverge only in their 5' UTR and encode the same protein (Adler *et al.*, 1988 and Takadera *et al.*, 1989). Lck expression is largely restricted to T cells and B cells, but has also been reported in colorectal carcinoma cells and small cell lung carcinomas (Veillette *et al.*, 1987; Adler *et al.*, 1988; Abts *et al.*, 1991; Nakamura *et al.*, 1996 and Krystal *et al.*, 1998). The proximal LCK type I promoter is activated in immature thymocytes and colon cancer cells, while the distal LCK type II promoter is predominantly expressed T and B cells (Sartor *et al.*, 1989; Leung *et al.*, 1993 and Nakamura *et al.*, 1996). Furthermore, malignant lymphocytes have been reported to use both the proximal and distal LCK promoters (Sartor *et al.*, 1989 and Leung *et al.*, 1993). Similar to the other SFK promoters, the LCK type I promoter is TATA-less and has more than one transcription start site (Leung *et al.*, 1993). Thymocyte maturation is principally regulated by two *cis*-acting elements in the

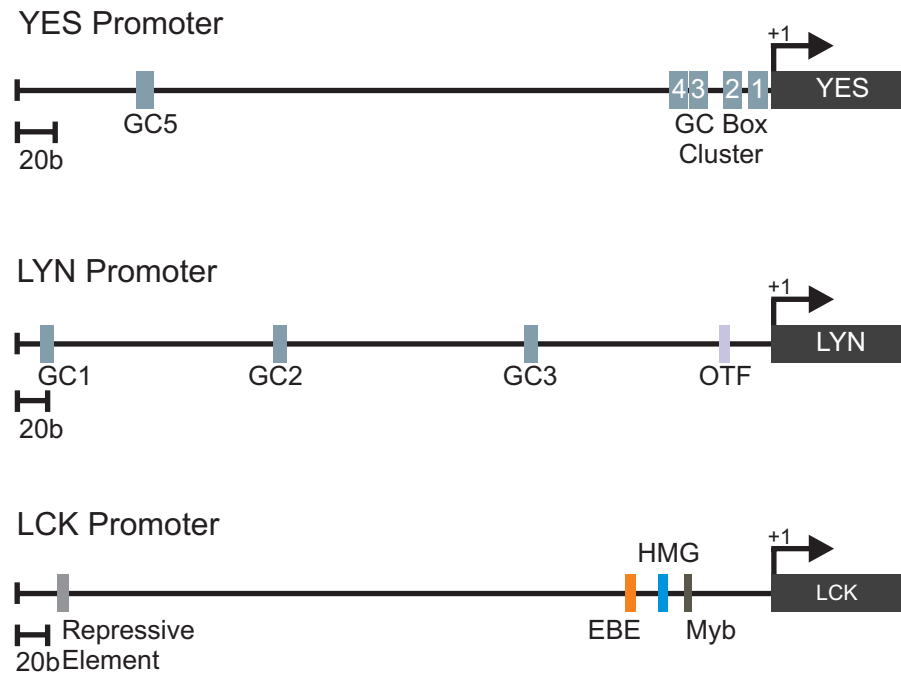


Figure 1.6. Gene promoters of human c-Src family kinase members. The proximal promoter *cis*-acting DNA binding elements are shown for the YES, LYN and LCK type I promoters. The YES promoter is regulated by five GC boxes, GC5 through GC1. LYN transcription is controlled by GC1, GC2, GC3, and an OTF motif. Activity of the LCK type I promoter is regulated by a repressive element, EBE and HMG consensus site in colorectal carcinoma cells. The EBE and Myb sites control LCK type I transcription in T cells. The arrows indicate the approximate transcription start sites. (Matsuzawa *et al.*, 1991, Uchiumi *et al.*, 1992, Leung *et al.*, 1993, McCracken *et al.*, 1994, Muise-Helmericks and Rosen, 1995 and McCracken *et al.*, 1997)

LCK type I promoter, an ETS binding element (EBE) and a Myb binding site, which interact with Ets1 or Ets2 and Myb transcription factors, respectively (Leung *et al.*, 1993 and McCracken *et al.*, 1994). Transactivation of the LCK type I promoter in colorectal carcinoma cells requires the EBE and a high mobility group (HMG) consensus binding site in the proximal promoter (McCracken *et al.*, 1997). The transcription factors Ets1 and Sox-4 have been reported to associate with these elements in colon cancer cells and cooperatively activate the LCK type I promoter (McCracken *et al.*, 1997). Furthermore, a *cis*-acting repressive element has also been identified in the LCK type I promoter that may control LCK transcription in colorectal cancer cells (Muise-Helmericks and Rosen, 1995).

## **1.6. p21WAF1**

In 1993, back to back reports were published in 'Cell' describing a novel protein that interacted with and inhibited cyclin dependent kinase (Cdk) activity, termed Cdk-interacting protein 1 (CIP1), as well as a gene product whose promoter was directly transactivated by the transcription factor p53, and so named wild-type p53-activated factor (WAF1) (Harper *et al.*, 1993 and el-Deiry *et al.*, 1993). Four months later, a third laboratory reported their discovery of a protein derived from senescent cells that inhibited DNA synthesis, labeled senescent cell-derived inhibitor 1 (sdi1) (Noda *et al.*, 1994). Interestingly, CIP1, WAF1 and sdi1 shared identical sequences and encoded the same 21 kDa protein, here on referred to as p21WAF1.

### **1.6.1. p21WAF1 Protein Function**

The cell cycle is regulated by the ordered activation and inactivation of cyclin-Cdk complexes. p21WAF1 is a cyclin dependent kinase inhibitor (CKI) that belongs to the Cip/Kip family and plays an important role in the regulation of cyclin-Cdk kinase activity. Through interactions with the catalytic subunits Cdk2, Cdk4 and Cdk6, p21WAF1 is involved in regulating the transition from G1 (Gap1) to S (synthesis) phase of the cell cycle (Harper *et al.*, 1995). At the start of the G1 phase, mitogenic signals stimulate transcription and translation of D-type cyclins (D1, D2, and D3), and promote their assembly into cyclin D-Cdk complexes with either the Cdk4 or Cdk6

catalytic subunit (Sherr and Roberts, 2004). Upon assembly, cyclin D-Cdk complexes are imported to the nucleus and activated by Cdk-activating kinases (CAK) (Sherr and Roberts, 1999). At the G1 midpoint, activated cyclin D-Cdk complexes, in succession with activated cyclin E-Cdk2 complexes, phosphorylate Rb family members (Coqueret, 2002). These phosphorylation events disrupt the interaction of Rb with E2F transcription factors and, therefore, relieve the inhibitory effect of Rb on E2F family members, enabling E2F activation of target genes in late G1 phase (Sherr and Roberts, 1999). Genes trans-activated by E2F include a cluster involved in DNA synthesis, as well as cyclin E and cyclin A2 (Sherr and Roberts, 2004). Newly assembled cyclin E-Cdk2 complexes prolong Rb phosphorylation, further enhancing the expression of E2F responsive genes (Sherr and Roberts, 2004). In addition, activated Cyclin D-Cdk4 and Cyclin E-Cdk2 complexes inhibit transforming growth factor beta (TGF- $\beta$ ) anti-proliferative signals by phosphorylating the Smad3 transcription factor, which prevents Smad3 transactivation of CKI genes, including p15 and p21WAF1 (Sherr and Roberts, 2004 and Liu and Matsuura, 2005). Cyclin E-Cdk2 and cyclin A2-Cdk2 complexes also facilitate entry into S-phase of the cell cycle by inducing phosphorylation of the CKI p27, marking it for proteasomal degradation (Sherr and Roberts, 2004).

p21WAF1 arrests the cell cycle in the G1 phase by inhibiting Cdk kinase activity of cyclinE-Cdk2 and cyclin A-Cdk2 complexes and averting Rb and Smad3 hyperphosphorylation (Sherr and Roberts, 2004 and Liu and Matsuura, 2005). In this manner, p21WAF1 is involved in both maintaining the repression of E2F-responsive genes and activating the transcription of Smad3 target CKIs. Additionally, p21WAF1 inactivates Cdk2 complexes, abolishing p27 phosphorylation and subsequent degradation, providing a tertiary means of inhibiting cell cycle progression beyond G1 (Sherr and Roberts, 1999 and Sherr and Roberts, 2004). However, in proliferating cells, p21WAF1 appears to be sequestered by cyclin D-Cdk complexes. While Cdk activity is not inhibited by this association, cyclin D-Cdk assembly, nuclear translocation and activation are promoted (Sherr and Roberts 1999 and Coqueret, 2002). At the same time, the pool of p21WAF1 available for cyclin E-Cdk2 and cyclin A2-Cdk2 binding is decreased (Sherr and Roberts 1999 and Coqueret, 2002). Therefore, p21WAF1 may be titrated by cyclin D-Cdk complexes, allowing the activation of Cdk2 complexes in the

late phase of G1 (Coqueret, 2002). Upon inhibition of cyclin D-Cdk by either CKI p16 or inactivation of mitogenic signals, cyclin D-Cdk complexes are disrupted and sequestered p21WAF1 is consequently released, promoting p21WAF1 association with cyclin E-Cdk2 and cyclin A2-Cdk2 and inhibition of the cell cycle (Sherr and Roberts, 1999). In addition to inducing G1 arrest, elevated p21WAF1 levels have been implicated as a contributing factor in S and G2/M phase arrest (Ogryzko *et al.*, 1997 and Niculescu *et al.*, 1998). Furthermore, p21WAF1 suppresses DNA synthesis and induces growth arrest by a supplementary mechanism, in which the activity of proliferating cell nuclear antigen (PCNA), a DNA polymerase delta processivity factor, is directly inhibited (Kelman, 1997). An additional role for p21WAF1 as a transcriptional cofactor has also been proposed, given that mutant p21WAF1 lacking the ability to bind either Cdks or PCNA is still able to suppress the expression of E2F-responsive genes (Perkins, 2002). p21WAF1 has been shown to transcriptionally upregulate the insulin-like growth factor I (IGF-I) gene and behaves as a function repressor of c-myc and cdc25 transcription upon association with these promoters (Devgan *et al.*, 2006 and Vigneron *et al.*, 2006). Taken together, p21WAF1 is an essential component of cell cycle, and an important regulator of cellular proliferation.

### **1.6.2. Regulation of p21WAF1**

The mechanisms facilitating p21WAF1 regulation have been intensively studied, along with their possible connections to cellular transformation, given that p21WAF1 is a critical modulator of the cell cycle arrest and an essential factor in the maintenance of normal cell growth. p21WAF1, itself, has rarely been found to harbor mutations in tumor cells, however, a key regulator of p21WAF1 expression has been explicitly implicated in human malignancies (Shiohara *et al.*, 1994 and Shiohara *et al.*, 1997). The tumor suppressor protein p53 is an important trans-activator of p21WAF1 transcription, mediating induction of the CKI and cell cycle arrest when met with cellular stress (el-Deiry *et al.*, 1993). In excess of 21,000 mutations have been reported in the p53 gene, with a large percentage of these residing in the DNA-binding domain, which prevents p53 mediated trans-activation of target genes (Liu and Chen, 2006 and Toledo and Wahl, 2006). Therefore, it has been suggested that tumors lacking wild-type p53 may

have compromised p21WAF1 expression levels. However, p21WAF1 may also be activated by p53 independent transcriptional mechanisms, including HDI stimulated transcription upon introduction of these anti-tumor agents (el Deiry *et al.*, 1994 and Nakano *et al.*, 1997). While p21WAF1 expression levels are relatively low in normal dividing cells, the regulation of p21WAF1 is primarily controlled at the transcriptional level by p53 dependent and p53 independent regulatory mechanisms (el Deiry *et al.*, 1994 and Gartel and Tyner, 1999). In addition, post-transcriptional mechanisms have also been reported to play a supporting role in the regulation of p21WAF1 levels (Gorospe *et al.*, 1998).

#### **1.6.2.1. p53 Dependent Regulation of p21WAF1 Transcription**

Loss of p53 function is evident in the majority of human neoplasms. While 50% of human cancers display mutations in the p53 gene itself, many other tumors exhibit alterations in p53 regulatory proteins, such as amplification or overexpression of the p53 regulatory factor, MDM2 (Baker *et al.*, 1989 and Toledo and Wahl, 2006). p53 is a well characterized tumor suppressor protein involved in stimulating cell cycle arrest, apoptosis, senescence and DNA repair upon cellular stresses, such as genotoxic stress (UV irradiation, carcinogens, cytotoxic drugs) induced DNA damage, non-genotoxic stress (physical strain, ribonucleotide depletion, hypoxia) and oncogenic stress (high growth signaling and oncogene expression) (Guimaraes and Hainaut, 2002; Bode and Dong, 2004 and Toledo and Wahl, 2006). Protein stabilization is the major regulatory mechanism of p53 expression, allowing rapid accumulation of p53 in response to cellular stress and the inhibition of damaged cell growth. Under normal or unstressed cellular conditions, p53 is a relatively unstable and inactive protein regulated by the E3 ubiquitin ligase activity of MDM2 and consequential 26S proteasome degradation. (Bode and Dong, 2004). However, upon cellular stress, p53 is stabilized and amasses in the nucleus, where it forms active homotetrameric transcription factor complexes that interact with and activate genes carrying p53 responsive elements, such as p21WAF1 (Bode and Dong, 2004). Post-translational modifications of the N-terminal transactivation domain, proline-rich domain and C-terminal regulatory domain of p53 have been proposed to regulate p53 stabilization and activity (Brooks and Gu, 2003 and Bode and Dong, 2004). In this model, unstressed cells maintain p53 in an inactive state

through MDM2 interaction with the N-terminal transactivation domain, while MDM2 ubiquitylation of the p53 C-terminal regulatory domain marks p53 for proteasome degradation (Brooks and Gu, 2003; Bode and Dong, 2004 and Toledo and Wahl, 2006). Conversely, post-translational modifications activate and stabilize p53 upon genotoxic cellular stress by inducing phosphorylation events within the N-terminal transactivation and proline-rich domains of p53 that disrupt MDM2 binding and enhance histone acetyltransferase association (Brooks and Gu, 2003; Bode and Dong, 2004 and Toledo and Wahl, 2006). p300 and CBP co-activator recruitment to the transactivation domain of p53 has been proposed to promote the acetylation of lysine residues in the C-terminal regulatory domain of p53, which consequently blocks ubiquitylation of these residues and possibly assists in histone acetylation of target genes (Brooks and Gu, 2003; Bode and Dong, 2004 and Toledo and Wahl, 2006). Furthermore, Set9 methylation of the p53 C-terminal domain has also been suggested to contribute to p53 stabilization by blocking MDM2 ubiquitylation (Liu and Chen, 2006). However, the role that MDM2 and post-translational mechanisms play in stabilizing p53 protein levels and activating p53 responsive genes may not be as simplistic nor as prominent as discussed (Guimaraes and Hainaut, 2002 and Toledo and Wahl, 2006). Nonetheless, p53 is an important regulator of p21WAF1 transcription and downstream p21WAF1 mediated growth arrest.

The p21WAF1 promoter contains two p53 responsive elements (RE), positioned at -2301 and -1384 bp relative to the transcription start site (Figure 1.7) (el Deiry *et al.*, 1993). Each of these REs contain two 10 bp sequences of 5' PuPuPuC(A/T)(T/A)Gpy PyPy-3', separated by a maximum of 13 bp (el Deiry *et al.*, 1993 and Gartel and Tyner, 1999). The p21WAF1 promoter is activated by p53 in response to genotoxic stress, such as caused by  $\gamma$ -ionizing radiation and doxorubicin, which induces DNA damage (el Deiry *et al.*, 1994). Resultant p21WAF1 induction has been suggested to be a definitive requirement for p53 facilitated G1 cell cycle arrest in tumor cells (Waldman *et al.*, 1995). In addition to DNA damaging events, upregulation of p53 and the consequent enhancement of p21WAF1 transcription also occur upon ribonucleotide depletion and enhanced Ras oncogene signaling (Gartel and Tyner, 1999). Therefore, part of the tumor suppressor function of p53 is mediated by p21WAF1 upregulation



when the cell is under various forms of stress. Hence, the absence of wild-type p53 in many human cancers may prevent cell cycle arrest in response to cellular stress.

#### **1.6.2.2. p53 Independent Regulation of p21WAF1 Transcription**

The p21WAF1 promoter is also activated in a p53 independent manner by a wide array of signaling events and downstream transcription factors (el Deiry *et al.*, 1994). The p21WAF1 proximal promoter contains a consensus TATA box and negotiates many p53 independent actions through six proximal Sp1 binding sites between -119 and -50 bp (Sp1-1: -119 to -114 bp, Sp1-2: -109 to -104 bp, Sp1-3: -82 to -76 bp, Sp1-4: -70 to -64 bp, Sp1-5: -60 to -53 bp, Sp1-6: -56 to -50 bp) (Figure 1.7.) (el Deiry *et al.*, 1993 and Gartel and Tyner, 1999). For example, in the absence of p53, the tumor suppressor protein breast cancer gene 1 (BRCA1) induces p21WAF1 expression through p53 independent transcription by mediating transactivation through a BRCA1 responsive element (BRE) located between -143 and -93 bp of the proximal promoter (Gartel and Tyner, 1999 and Somasundaram, 2003). In contrast, the differentiating agents phorbol myristate acid (PMA) and okadaic acid (OA) mediate p21WAF1 transcription through Sp1 association with the Sp1-1 and Sp1-2 binding elements (Gartel and Tyner, 1999). HDIs have been reported to upregulate p21WAF1 transcription via the Sp1-3 and Sp1-4 sites of the promoter (Nakano *et al.*, 1997). Androgen stimulation of p21WAF1 requires androgen receptor interaction with both the androgen response element and Sp1 at the Sp1-3 site in the p21WAF1 proximal promoter (Lu *et al.*, 1999 and Lu *et al.*, 2000). Furthermore, progesterone controls activation of the p21WAF1 promoter by progesterone receptor binding of Sp1 linked to the Sp1-3 and Sp1-4 binding elements, in cooperation with CBP/p300 (Owen *et al.*, 1998 and Gartel and Tyner, 1999). The p21WAF1 promoter is also activated in response to TGF- $\beta$  signaling, via binding of Smad3 and Smad4 proteins to Sp1, which enhances the affinity of Sp1 for the Sp1-3 site of the p21WAF1 proximal promoter (Pardali *et al.*, 2000). TGF- $\beta$  also stimulates p21WAF1 transcription by inducing the expression of c-jun, a known Sp1 superactivator (Kardassis *et al.*, 1999). As well, four E2F transcription factor binding elements and an activator protein 2 (AP2) binding motif also exist in the p21WAF1 proximal promoter that regulate transcription (Gartel and Tyner, 1999).

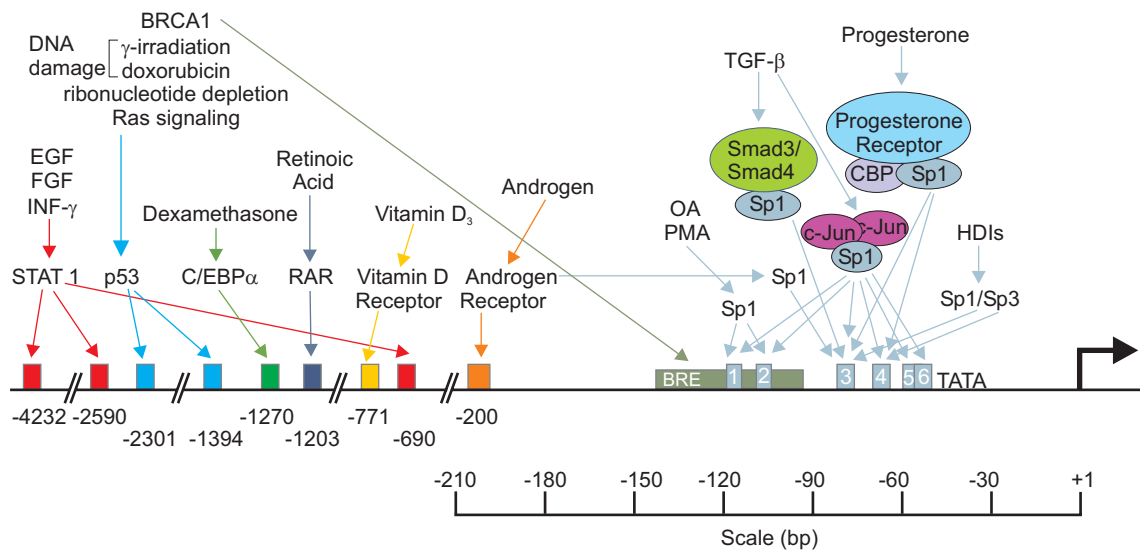


Figure 1.7. Regulation of the p21WAF1 promoter. Various *cis*-acting responsive elements and proximal promoter elements mediate transactivation of the p21WAF1 promoter in response to cellular stress (DNA damage, ribonucleotide depletion, oncogenic signaling), tumor suppressor genes (p53, breast cancer gene 1 - BRCA1), growth factor signaling (epidermal growth factor - EGF, fibroblast growth factor - FGF, transforming growth factor beta -TGF-β), hormones (dexamethasone, retinoic acid - RA, vitamin D<sub>3</sub>, androgen, progesterone), differentiation factors (phorbol myristate acetate - PMA, okadaic acid - OA), and histone deacetylase inhibitors - HDIs. The Sp1 binding sites are numbered 1-6. (Gartel *et al.*, 1999 and Gartel *et al.*, 2005)

In addition, the p21WAF1 promoter contains *cis*-regulatory elements beyond the proximal promoter region responsible for activating transcription (Figure 1.7) (Gartel and Tyner, 1999). To name a few, Vitamin D<sub>3</sub> stimulates the p21WAF1 promoter through a vitamin D responsive element at -771 bp, and a retinoic acid responsive element has been characterized at -1203 bp that facilitates RA activation of p21WAF1 (Liu *et al.*, 1996a and Liu *et al.*, 1996b). A DNA binding element for CCAAT/enhancer binding protein- $\alpha$  (C/EBP $\alpha$ ), which resides at -1270 bp, is required for dexamethasone activation of the p21WAF1 promoter (Cha *et al.*, 1998 and Gartel and Tyner, 1999). Furthermore, three STAT binding sites are present at -690, -2590, and -4233 bp, which mediate epidermal growth factor (EGF), fibroblast growth factor (FGF), and IFN- $\gamma$  activation of the p21WAF1 promoter by STAT1 (Chin *et al.*, 1996 and Gartel and Tyner, 1999). Thus, the p21WAF1 promoter is activated through various *cis*-acting elements by an array of factors, including cellular stress, tumor suppressor genes, differentiation agents, hormones and growth factors (Kardassis *et al.*, 1999).

#### **1.6.2.3. Histone Deacetylase Inhibitor Regulation of p21WAF1/Cip1 Transcription**

As HDIs are such promising chemotherapeutic agents, and p21WAF1 has been thoroughly implicated as an inducer of cell cycle arrest, the role that p21WAF1 may play in mediating the anti-tumor effects of HDIs has been extensively investigated. Early studies into the involvement of p21WAF1 in HDI facilitated cell cycle arrest, differentiation and apoptosis identified that p21WAF1 was an absolute requirement for G1 growth arrest in response to butyrate in colon cancer cells (Archer *et al.*, 1998). Furthermore, parallel observations have since been documented upon butyrate and MS-275 treatment in myelomonocytic leukemia cells (Rosato *et al.*, 2001 and Rosato *et al.*, 2003). Hence, as a consequence of this information, the cyclin dependent kinase inhibitor p21WAF1 has easily become the most extensively studied HDI responsive gene.

Reports by Toshiyuki Sakai's group have provided the foundation of HDI mediated p21WAF1 regulation, outlining that the HDIs butyrate, TSA and SAHA induced p21WAF1 transcription independent of p53 and free of new protein synthesis (Nakano *et al.*, 1997; Sowa *et al.*, 1997 and Huang *et al.*, 2000). HDI transcriptional activation of the p21WAF1 promoter was reported to chiefly require the third Sp1

binding element upstream of the TATA box, Sp1-3, while the Sp1-4 site also appeared to display involvement (Figure 1.7.) (Nakano *et al.*, 1997 and Sowa *et al.*, 1997). Furthermore, both Sp1 and Sp3 were identified to interact with these Sp1 sites, and while the binding affinity of neither transcription factor changed upon TSA or SAHA treatment, the transactivation domain of Sp3, but not Sp1 was found to mediate TSA upregulation of p21WAF1 transcription (Sowa *et al.* 1999 and Huang *et al.* 2000). Sp1 and Sp3 were, however, reported to facilitate SAHA induced transcription of the p21WAF1 promoter (Huang *et al.*, 2000). In addition to these observations, Sakai also noted prominent increases in global H4 acetylation levels and growth arrest at the G1 and G2/M phases of the cell cycle in association with TSA treatment (Sowa *et al.*, 1997). Similarly, other groups reported that HDIs, including MS-275 and apicidin, increased p21WAF1 transcription independent of p53 and *de novo* protein synthesis, as well as enhanced global histone H3 and/or H4 acetylation and the percentage of cells in G1 cell cycle arrest (Saito *et al.*, 1999; Han *et al.*, 2000 and Han *et al.*, 2001). Furthermore, the advent of the chromatin immunoprecipitation (ChIP) assay revealed that overall H3 and H4 acetylation levels were selectively induced in the chromatin associated with the p21WAF1 promoter and downstream gene, upon treatment with SAHA and apicidin (Richon *et al.*, 2000 and Kim *et al.*, 2001). This suggested that local histone acetylation may regulate HDI activation of selective genes, such as p21WAF1. Fueling greater support of this theory, p300 was implicated in TSA stimulation of the p21WAF1 promoter via the Sp1-4 element, while HDAC1, HDAC2 and HDAC3, which are capable of attenuating butyrate induced p21WAF1 transactivation, were conversely identified as repressors of p21WAF1 transcription (Xiao *et al.*, 2000; Lager *et al.*, 2003; Gui *et al.*, 2004; Huang *et al.*, 2005 and Wilson *et al.*, 2006). Taken together, HDIs have been proposed to induce cell cycle inhibition via p21WAF1 transcriptional activation as a consequence of gene hyperacetylation, subsequent chromatin remodeling and transcription factor recruitment.

However, histone hyperacetylation of the p21WAF1 gene may not fully justify transcriptional activation. A report has identified protein kinase C (PKC) as an essential signaling molecule associated with butyrate, TSA and apicidin driven p21WAF1 upregulation (Han *et al.*, 2001). More specifically, a signaling pathway that proposes

phosphatidylinositol 3-kinase (PI 3-kinase) as the upstream effector of the PCK epsilon isoform has been elucidated. The PCK $\epsilon$  isoform then, via the Sp1-3 site of the p21WAF1 promoter facilitates apicidin induced p21WAF1 expression, (Han *et al.*, 2001 and Kim *et al.*, 2003). Apicidin treatment has also been shown to enhance histone H3 acetylation levels in the p21WAF1 promoter (Han *et al.*, 2000 and Han *et al.*, 2001). Treatment with either a PI 3-kinase or PKC inhibitor in addition to apicidin, however, reduced p21WAF1 promoter expression without decreasing apicidin induced H3 acetylation, thereby suggesting that histone hyperacetylation and chromatin remodeling are inadequate for p21WAF1 activation (Kim *et al.*, 2003). PI 3-kinase activity has also been independently identified as a prerequisite for HDI transactivation of p21WAF1 in the study of Ataxia telangiectasia disorder (Ju and Muller, 2003). Furthermore, another report suggests that HDI mediated downregulation and release of c-myc from the p21WAF1 proximal promoter may play a contributing role in p21WAF1 gene induction, as c-myc is a known repressor of p21WAF1 (Gui *et al.*, 2004 and Li and Wu, 2004). In addition, the HDI depsipeptide has been documented to enhance p21WAF1 promoter activity by p53 independent and p53 dependent mechanisms, where p53 acetylation at K373/K382 promoted a p53 protein longer half-life and enhanced DNA binding affinity of p53 to the p21WAF1 promoter (Zhao *et al.*, 2006). Therefore, the mechanism of HDI mediated growth arrest via p21WAF1 upregulation likely requires extra events beyond enhancing histone acetylation levels.

#### **1.6.2.4. p21WAF1 mRNA stability**

While regulation of p21WAF1 expression levels has been prominently documented to arise from transcriptional mechanisms, control of p21WAF1 mRNA stability should not be disregarded as an additional p53 independent regulatory means. To date, many cellular agents have been reported to induce p21WAF1 mRNA stability. For example, PMA and Vitamin D3 mediate differentiation of human promyelocytic cells into monocytes by enhancing p21WAF1 mRNA stability, without affecting the rate of p21WAF1 transcription (Schwaller *et al.*, 1995). Furthermore, p21WAF1 mRNA stability is also upregulated by: novel retinoid CD437 prolonged stimulation of the p42/44 mitogen-activated protein kinase pathway, amino acid depletion, ultraviolet light (UVC) irradiation, prostaglandin A<sub>2</sub>, the  $\alpha_1$ -adrenergic receptor agonist phenylephrine,

and EGF (Li *et al.*, 1996; Gorospe *et al.*, 1998; Johannessen *et al.*, 1999; Liu *et al.*, 2000; Park *et al.*, 2000; Giles *et al.*, 2003; Leung-Pineda *et al.*, 2004 and Yang *et al.*, 2004).

Investigation into the mechanism underlying p21WAF1 post-transcriptional regulation has focused on *cis*-acting stability determinants within the 3' UTR of the p21WAF1 mRNA and the identification of *trans*-associated RNA binding proteins (Figure 1.8.). Importantly, the p21WAF1 3'UTR (corresponding to nucleotides 571 through 2102 of the mRNA) contains three highly destabilizing AREs, termed A (742 to 759 ntd), B (798 to 810 ntd) and C (812 to 825 ntd), as well as a 42 nucleotide HuD binding element (658 to 700 ntd) located within a U-rich tract (el-Deiry *et al.*, 1993 and Joseph *et al.*, 1998). UVC mediated stabilization of p21WAF1 mRNA levels provided the first direct evidence for the involvement of the p21WAF1 3' UTR in mRNA stabilization, as UVC was shown to trigger an interaction between cytoplasmic HuR and a fragment of the 3' UTR, which mapped to the proximal 3' UTR nucleotide sequence 571 to 851 (Wang *et al.*, 2000). An additional report specified that UVC caused a decrease in the association of AUF1 with the 3' UTR and enhanced the interaction of HuR with the p21WAF1 mRNA, which ultimately led to an overall increase in the abundance of HuR-bound p21WAF1 mRNA in the polysomal fraction (Lal *et al.*, 2004). As well, Poly(C) binding protein, CP1, has also been documented to interact with the ribonucleotide sequence between 571 to 829 and may potentially play a role in p21WAF1 mRNA stabilization upon UVC exposure (Giles *et al.*, 2003). Similar to what happens following UVC exposure, HuR interacts with nucleotides 570 to 830 of the p21WAF1 3' UTR and stabilizes p21WAF1 mRNA levels following prostaglandin A<sub>2</sub> treatment (Yang *et al.*, 2004). Furthermore, nucleotides 630-707 of the p21WAF1 3' UTR have also been implicated in mRNA stabilization and interact with an unidentified *trans*-RNA binding protein in the presence of phenylephrine (Liu *et al.*, 2000). Therefore, the first 300 bases of the p21WAF1 3' UTR appear to contain important determinants of p21WAF1 mRNA stability. However, evidence also exists for yet uncharacterized p21WAF1 3' UTR stability determinants downstream of the HuD and ARE elements, as removal of the proximal region of the 3' UTR still results in rapid mRNA destabilization (Li *et al.*, 1996 and Giles *et al.*, 2003). Furthermore, EGF has

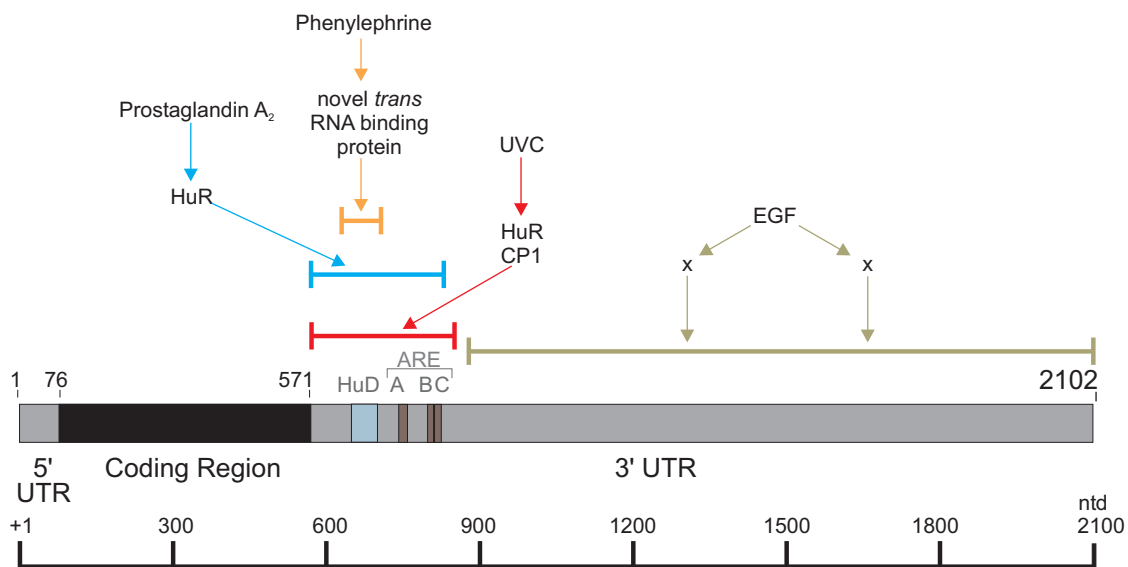


Figure 1.8. The p21WAF1 mRNA and the *trans*-acting RNA binding proteins that regulate p21WAF1 mRNA stability in response to various agents. Nucleotides 1-75 encompass the 5' UTR, 76-570 the coding region and 571-2102 the 3' UTR. The HuD and ARE (A, B, and C) *cis*-acting determinants are shown in the 3' UTR. Regions of the 3' UTR and the *trans*-acting factors important for mRNA stabilization of p21WAF1 upon exposure of UVC, prostaglandin A<sub>2</sub>, phenylephrine and EGF are denoted above the mRNA. X represents yet to be identified *trans*-associated RNA binding proteins.

recently been reported to regulate p21WAF1 mRNA stability through multiple *cis*-acting determinants associated with the mRNA sequence between nucleotides 879 and 2102 of the 3' UTR (Giles *et al.*, 2003). Therefore the 3' UTR of the p21WAF1 mRNA contains important mRNA stability determinants.



## 2. SPECIFIC AIMS AND HYPOTHESIS

Histone deacetylase inhibitors (HDIs) are impressive anti-tumor agents that elicit a wide range of regulatory actions in malignant cells. The HDI butyrate is a naturally manufactured product of dietary fiber in the colon responsible for the maintenance of normal colonic mucosa growth. Conversely, butyrate invokes cell cycle arrest, differentiation, and/or apoptosis in tumor cells, suggesting that high fiber diets may have a chemo-preventive effect against colonic malignancies. Interestingly, elevated expression levels and/or kinase activity of the proto-oncogene c-Src and the c-Src family kinase (SFK) members have been extensively reported in human neoplasms, in particular those of the colon. Furthermore, downregulation of c-Src decreases tumor cell growth and tumorigenicity, suggesting that c-Src may be an important therapeutic target in the treatment of carcinomas expressing high levels of c-Src.

The mechanisms of HDI action are not well understood. HDIs have characteristically been associated with histone acetylation coupled to transcriptional activation. The cyclin dependent kinase inhibitor p21WAF1 has served as the model gene for HDI mediated regulation, although transcriptional repression of a number of genes has also been observed following treatment with these drugs. Furthermore, HDIs have been implicated in non-histone acetylation and the alteration of phosphorylation status. We, therefore, sought to investigate HDI mediated gene regulation in tumor cells.

**HYPOTHESIS:** Histone deacetylase inhibitors regulate the expression levels of the c-Src family kinase members and p21WAF1 by alternative mechanisms, in relevance to cancer.

**SPECIFIC AIMS:**

- 1) To examine the effects of butyrate on the expression levels of c-Src and the c-Src family kinase members in colon cancer cells.
- 2) To identify the mechanism of histone deacetylase inhibitor stimulated p21WAF1 upregulation in hepatocellular carcinoma cells.

### 3. MATERIALS AND METHODS

#### 3.1. Reagents, Equipment, Software and Distributors

The distributors of the reagents and commercially available kits used throughout these studies are detailed in Table 3.1. and 3.2., respectively. Table 3.3. outlines the equipment utilized and product distributor. The distributor names and addresses are presented in Table 3.4. All primers and oligonucleotides presented in this thesis are documented in Table 3.5. The underlined nucleotides represent the sequence to be mutated and the nucleotides shown in bold constitute the poly(A) signal. The primers and the oligonucleotides under 100 nucleotides were purchased from Invitrogen. Those oligonucleotides greater than 100 nucleotides were obtained from Integrated DNA Technologies (IDT).

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**Table 3.1. The List of Reagents and Distributors**

Reagents and Materials	Distributor Name
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Sigma-Aldrich Co.
[ $\alpha$ - <sup>32</sup> P] dCTP	PerkinElmer Life Sciences Inc.
[ $\alpha$ - <sup>32</sup> P] rUTP	PerkinElmer Life Sciences Inc.
Acetic acid (glacial)	BDH
Acrylamide	Sigma-Aldrich Co.
Actinomycin D	Sigma-Aldrich Co.
Agarose	EMD Chemicals Inc.
Ammonium persulfate	EMD Chemicals Inc.
Ampicillin	EMD Chemicals Inc.
Anti-v-Src (Ab-1) mouse monoclonal antibody	Calbiochem
Bis-Acrylamide	Sigma-Aldrich Co.
$\beta$ -Mercaptoethanol	EMD Chemicals Inc.

Bacto Agar	BD Biosciences
BioMax XAR film	Kodak
Boric acid	EMD Chemicals Inc.
Bromophenol blue	BDH
Calcium chloride dihydrate	BDH
Calf Intestinal Alkaline Phosphatase	New England Biolabs Ltd.
Chloroform	EMD Chemicals Inc.
CMV c-jun	ATCC
Cycloheximide	Sigma-Aldrich Co.
Diethyl pyrocarbonate	Sigma-Aldrich Co.
Disodium hydrogen phosphate	EMD Chemicals Inc.
Dithiothreitol	Fermentas Canada Inc.
DMEM	Invitrogen – Gibco Cell Culture Systems
DMEM-F12	Invitrogen – Gibco Cell Culture Systems
DMSO	Sigma-Aldrich Co.
dNTPs	New England Biolabs Ltd.
Dodecyl sodium sulfate	EMD Chemicals Inc.
EDTA	EMD Chemicals Inc.
Ethidium bromide	BDH
Fat-free skim milk powder	Carnation
Fetal Bovine Serum	HyClone
Formaldehyde	EMD Chemicals Inc.
Formamide	BDH
Fyn EST (7997662)	ATCC
GeneScreen Plus Hybridization Transfer Membrane	PerkinElmer Life Sciences, Inc.
Glycerol	EMD Chemicals Inc.
Glycine	EMD Chemicals Inc.
goat anti-mouse IgG-HRP SC-2005	Santa Cruz Biotechnology
Guanidinium thiocyanate	BDH
Humulin	Eli Lilly Canada Inc.
Hydrochloric acid	EMD Chemicals Inc.
Isoamyl alcohol	BDH
Isopropanol	EMD Chemicals Inc.
Kanamycin	EMD Chemicals Inc.
Klenow Fragment	Fermentas Canada Inc.
Lambda DNA	GE Healthcare Bio-Sciences Inc.
Lck EST (6883710)	ATCC

Lyn EST (5509761)	ATCC
Manganese chloride tetrahydrate	BDH
Magnesium chloride	BDH
Molecular Imager FX Imaging Screen K	Bio-Rad Laboratories Ltd.
Morpholinopropanesulfonic acid	Sigma-Aldrich Co.
N-Lauroyl sarcosine	Sigma-Aldrich Co.
N,N,N',N'- Tetramethylethylenediamine	EMD Chemicals Inc.
Nonidet-P-40	Sigma-Aldrich Co.
O'GeneRuler 50 bp DNA Ladder	Fermentas Canada Inc.
O'GeneRuler 1 kb DNA Ladder	Fermentas Canada Inc.
o-nitrophenyl- $\beta$ -D-galactopyranoside	Sigma-Aldrich Co.
Optitran Supported Nitrocellulose	Schleicher & Schuell
p21 (187) sc-817 mouse monoclonal antibody	Santa Cruz Biotechnology
pBluescript KS +	Stratagene
pCAT3-Basic	Promega
penicillin-streptomycin	Invitrogen – Gibco Cell Culture Systems
<i>Pfu</i> DNA Polymerase	Fermentas Canada Inc.
Phenol (water saturated)	EMD Chemicals Inc.
Phenylmethylsulphonyl fluoride	GE Healthcare Bio-Sciences Inc.
Potassium Acetate	BDH
Potassium Chloride	EMD Chemicals Inc.
Potassium dihydrogen phosphate	EMD Chemicals Inc.
Potassium hydroxide	BDH
Protease Inhibitor Mix	Sigma-Aldrich Co.
Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories Ltd.
Rubidium Chloride	EMD Chemicals Inc.
Restriction endonucleases	New England Biolabs Ltd.
RPMI-1640	Invitrogen – Gibco Cell Culture Systems
Shrimp Alkaline Phosphatase	Fermentas Canada Inc.
Sodium acetate	BDH
Sodium butyrate	Sigma-Aldrich Co.
Sodium bicarbonate	EMD Chemicals Inc.
Sodium carbonate	EMD Chemicals Inc.
Sodium chloride	EMD Chemicals Inc.
Sodium citrate	BDH

Sodium dihydrogen phosphate	BDH
Sodium dodecyl sulfate	EMD Chemicals Inc.
Sodium hydroxide	BDH
Sodium phosphate (monobasic)	EMD Chemicals Inc.
Sodium phosphate (dibasic)	EMD Chemicals Inc.
SuperFect Transfection Reagent	Qiagen Inc.
T4 DNA Polymerase	GE Healthcare Bio-Sciences Inc.
<i>Taq</i> DNA Polymerase	Qiagen Inc.
Trichostatin A	Sigma-Aldrich Co.
Tris	EMD Chemicals Inc.
Trypan blue stain	Invitrogen – Gibco Cell Culture Systems
Trypsin-EDTA 1X	Invitrogen – Gibco Cell Culture Systems
TWEEN-20	Sigma-Aldrich Co.
Western Lightening Chemiluminescence Reagent Plus	PerkinElmer Life Sciences, Inc.
X-OMAT Blue XB-1 film	Kodak
Xylene Cyanol FF	BDH
Yes EST A50(7497767)	ATCC

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**Table 3.2. Commercial Kits and Distributors**

<b>Commercial Kit</b>	<b>Distributor Name</b>
CAT ELISA Kit	Roche Diagnostics
EndoFree Plasmid Maxi Kit	Qiagen Inc.
FastPlasmid Mini Kit	Eppendorf AG
QIAfilter Plasmid Midi Kit	Qiagen Inc.
QIAprep Miniprep Kit	Qiagen Inc.
QIAquick Gel Extraction Kit	Qiagen Inc.
Quick Ligation Kit	New England Biolabs, Ltd.
Riboprobe <i>in vitro</i> Transcription System	Promega
RNeasy Mini Kit	Qiagen Inc.
Total Protein Kit, Micro Lowry	Sigma-Aldrich Co.
Zero Blunt TOPO PCR Cloning Kit	Invitrogen

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**Table 3.3. Equipment & Software and Distributors**

<b>Equipment and Software</b>	<b>Distributor Name</b>
Biofuge 13 microcentrifuge	Thermo Electron Corporation - Heraeus
CO <sub>2</sub> Incubator 3326	Forma Scientific, Inc.
Coulter Counter ZM	Coulter Electronics, Ltd.
Gel Doc 2000	Bio-Rad Laboratories Ltd.
Gel Dryer Model 583	Bio-Rad Laboratories Ltd.
Gene Amp PCR System 2700	Applied Biosystems Canada
Horizontal Gel Electrophoresis System	Owl Separation Systems
Isotemp Incubator 230D	Fisher Scientific Company
JA-10 rotor	Beckman Coulter Canada, Inc.
J2-MI highspeed centrifuge	Beckman Coulter Canada, Inc.
MacVector 7.2.3	Accelrys Inc.
Micro Hybridization Incubator Model 2000	Robbins Scientific
Microplate Reader Model 3550	Bio-Rad Laboratories Ltd.
Mini-PROTEAN II Cell	Bio-Rad Laboratories Ltd.
Molecular Imager FX	Bio-Rad Laboratories Ltd.
ORBIT Incubator Shaker	BarnsteadIThermolyne Corp. International
PROTEAN II xi Cell	Bio-Rad Laboratories Ltd.
SmartSpec 3000 Spectrophotometer	Bio-Rad Laboratories Ltd.
Sorvall RT6000D	Du Pont Canada, Inc.
Quantity One Software, Version 4	Bio-Rad Laboratories Ltd.
UV Stratalinker 2400	Stratagene

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**Table 3.4. Distributor Addresses**

<b>Distributor Name</b>	<b>Address</b>
Accelrys Inc.	Accelrys Inc., San Diego, CA, USA
Applied Biosystems Canada	Applied Biosystems Canada, Streetsville, ON, Canada
ATCC (American Type Culture Collection)	American Type Culture Collection, Manassas, VA, USA
BarnsteadIThermolyne Corp. International	BarnsteadIThermolyne Corp., Dubuque, Iowa, USA
BD Biosciences	BD Biosciences, Mississauga, ON, Canada
BDH Inc.	BDH Inc., Toronto, ON, Canada

Beckman Coulter Canada, Inc.	Beckman Coulter Canada, Inc., Mississauga, ON, Canada
Bio-Rad Laboratories Ltd.	Bio-Rad Laboratories Ltd., Mississauga, ON, Canada
Calbiochem	EMD Biosciences Inc., La Jolla, CA, USA
Coulter Electronics Ltd.	Coulter Electronics Ltd. Luton, Beds., England
Du Pont Canada, Inc.	Du Pont Canada, Inc., Mississauga, ON, Canada
Eli Lilly Canada Inc.	Eli Lilly Canada Inc., Scarborough, ON, Canada
EMD Chemicals Inc.	EMD Chemicals Inc., Gibbstown, NJ, USA
Eppendorf AG	Eppendorf AG, Hamberg, Germany
Fermentas Canada Inc.	Fermentas Canada Inc., Burlington, ON, Canada
Fisher Scientific Company	Fisher Scientific Company, Nepean, ON, Canada
Forma Scientific, Inc.	Forma Scientific, Inc., Marietta, OH, USA
GE Healthcare Bio-Sciences Inc.	GE Healthcare Bio-Sciences Inc., Baie d'Urfe, QC, Canada
HyClone	HyClone, Logan, UT, USA
Integrated DNA Technologies, Inc.	Integrated DNA Technologies, Inc., Coralville, IA, USA
Invitrogen Canada Inc.	Invitrogen Canada Inc., Burlington, ON, Canada
Kodak	Eastman Kodak Company, Rochester, NY, USA
New England Biolabs, Ltd.	New England Biolabs, Ltd., Pickering, ON, Canada
Owl Separation Systems	Owl Separation Systems, Portsmouth, NH, USA
PerkinElmer Life Sciences, Inc.	PerkinElmer Life Sciences, Inc., Boston, MA, USA
Promega	Promega, Madison, WI, USA
Qiagen Inc.	Qiagen Inc., Mississauga, ON, Canada
Robbins Scientific	Robbins Scientific, Sunnyvale, CA, USA
Roche Diagnostics	Roche Diagnostics, Laval, QC, Canada
Santa Cruz Biotechnology Inc.	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA



Schleicher & Schuell Inc.	Schleicher & Schuell Inc., Keene, New Hampshire, USA
Sigma-Aldrich Co.	Sigma-Aldrich Co., Oakville, ON, Canada
Stratagene	Stratagene, La Jolla, CA, USA
Thermo Electron Corporation - Heraeus	Thermo Electron Corporation, Waltham, MA, USA

**Table 3.5. PCR Primers and Oligonucleotides**

Name	Sequence	Description
FWD LCK KpnI -634	5' CATGGTACCTTAACTTTGAGG AC AGCGCTTTCC 3'	PCR primer
REV LCK XhoI +145	5' CTCCCTCGAGATCTGCTCCCCCA ACATCAG 3'	PCR primer
FWD LYN -593	5' CCCGGTACCGAAAGGTACACG 3'	PCR primer
REV LYN +14	5' CGGCTCGAGAGGAAGTGGCTG 3'	PCR primer
FWD p21WAF1 3'UTR 591	5' GGAAGTCTAGAGTCCTGGAAG 3'	PCR primer
REV p21WAF1 3'UTR 2079	5' CGTTTCTAGAGCACCTGCTG 3'	PCR primer
FWD p21WAF1 3'UTR 712	5' CATACTCTAGACGCCCCCTGC 3'	PCR primer
FWD p21WAF1 3'UTR 788	5' GAGGTTTCTAGAAGTGCTGGGC 3'	PCR primer
FWD p21WAF1 3'UTR 840	5' CCTCATCTAGAGTTCTCCTTTTCC 3'	PCR primer
REV p21WAF1 3'UTR 840	5' GAGAACTCTAGATGAGGAGGC 3'	PCR primer
FWD p21WAF1 3'UTR 932	5' GTACCCTCTAGAGGGGTGTGGCT C 3'	PCR primer
FWD p21WAF1 3'UTR 1045	5' GGCATCTAGAAGGGGCCTCAC 3'	PCR primer
FWD p21WAF1 3'UTR 1173	5' GTACCCTCTAGACTCTTGATACC 3'	PCR primer
FWD p21WAF1 3'UTR 1272	5' CCTGCTCTAGAGAGCCCGTCTC 3'	PCR primer
REV p21WAF1 3'UTR 1470	5' CCCCTTCTAGACCATGAGCTGG G 3'	PCR primer
REV p21WAF1 3'UTR 2064	5' CCTGCTCTAGATTGAGCATTG 3'	PCR primer
REV p21WAF1 3'UTR 2031	5' GAAAGATCTAGAACAGTACAGG 3'	PCR primer
REV p21WAF1 3'UTR 2000	5' GCCCTCTAGAGGTTTACAGTC 3'	PCR primer
REV p21WAF1 3'UTR 1979	5' CTAGGTCTAGAAACGGGAACCA GG 3'	PCR primer
FWD p21WAF1 3'UTR 1467	5' CCCCATCTAGATGGACTGGAAGG 3'	PCR primer

FWD p21WAF1 3'UTR (T <sub>7</sub> )586	5' CCAAGCTTCTAATACGACTCACT ATAGGGAGACCTGCAGTCCTGGAAGC GCGAGG 3'	PCR primer
FWD p21WAF1 3'UTR (T <sub>7</sub> )835	5' CCAAGCTTCTAATACGACTCACT ATAGGGAGACCCGTGTTCTCCTTTT CCTCTC 3'	PCR primer
FWD p21WAF1 3'UTR (T <sub>7</sub> )1459	5' CCAAGCTTCTAATACGACTCACT ATAGGGAGACCAGCTCAATGGACT GGAAGGG 3'	PCR primer
FWD -210WAF1 XbaI destroy	5' GCGGGGCGTAATCTGCAGTCCTGG AAGCGCG 3'	Site - directed mutagenesis primer
REV -210WAF1 XbaI destroy	5' CGCGCTTCCAGGACTGCAGATTA CGCCCCGC 3'	Site - directed mutagenesis primer
FWD WAF1 3'UTR EcoRV create	5' CTCCACCTAGACTGATATCCTCTC GAGGGCAGG 3'	Site - directed mutagenesis primer
REV WAF1 3'UTR EcoRV create	5' CCTGCCCTCGAGAGGATATCAGTC TAGGTGGAG 3'	Site - directed mutagenesis primer
sense WAF1 3'UTR mut #1	5' ATCCTCTTAGAAGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #1 complementary oligonucleotide
antisense WAF1 3'UTR mut #1	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTGAAAG ACACAGAACAGTACAGGGTGTGGT CCCTGCTTCTAAGAGGAT 3'	cloning - mut #1 complementary oligonucleotide
sense WAF1 3'UTR mut #2	5' ATCCTCTCGAGGATGAAGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #2 complementary oligonucleotide
antisense WAF1 3'UTR mut #2	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTGAAAG ACACAGAACAGTACAGGGTGTGGT CTTCATCCTCGAGAGGAT 3'	cloning - mut #2 complementary oligonucleotide
sense WAF1 3'UTR mut #3	5' ATCCTCTCGAGGGCAGGAGTTGC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #3 complementary oligonucleotide
antisense WAF1 3'UTR mut #3	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTGAAAG ACACAGAACAGTACAGGGTGCAA CTCCTGCCCTCGAGAGGAT 3'	cloning - mut #3 complementary oligonucleotide
sense WAF1 3'UTR mut #4	5' ATCCTCTCGAGGGCAGGGACCAT GTTTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #4 complementary oligonucleotide

antisense WAF1 3'UTR mut #4	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTGAAAG ACACAGAACAGTACAAAACATGG TCCCTGCCCTCGAGAGGAT 3'	cloning - mut #4 complementary oligonucleotide
sense WAF1 3'UTR mut #5	5' ATCCTCTCGAGGGCAGGGACCAC ACCCACGTTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #5 complementary oligonucleotide
antisense WAF1 3'UTR mut #5	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTGAAAG ACACAGAACAACGTGGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #5 complementary oligonucleotide
sense WAF1 3'UTR mut #6	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACACCTTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #6 complementary oligonucleotide
antisense WAF1 3'UTR mut #6	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTGAAAG ACACAAGGTGGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #6 complementary oligonucleotide
sense WAF1 3'UTR mut #7	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCCACACCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #7 complementary oligonucleotide
antisense WAF1 3'UTR mut #7	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTGAAAG GTGTGGAACAGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #7 complementary oligonucleotide
sense WAF1 3'UTR mut #8	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTTCCCTA CAGCTCCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #8 complementary oligonucleotide
antisense WAF1 3'UTR mut #8	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTAGGGA ACACAGAACAGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #8 complementary oligonucleotide
sense WAF1 3'UTR mut #9	5' ATCCTCTCGAGGGCAGGGACCACA CCCTGTACTGTTCTGTGTCTTTCTG ATTCTCCACAATGCTGAATATA CAGCAGGTGCT 3'	cloning - mut #9 complementary oligonucleotide
antisense WAF1 3'UTR mut #9	5' CTAGAGCACCTGCTGTATATTCA GCATTGTGGGAGGAATCACGAAAG ACACAGAACAGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #9 complementary oligonucleotide
sense WAF1 3'UTR mut #10	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCCTTCTCCACAATGCTGAATA TACAGCAGGTGCT 3'	cloning - mut #10 complementary oligonucleotide

antisense WAF1 3'UTR mut #10	5' CTAGAGCACCTGCTGTATATTC AGCATTGTGG <u>AGAAG</u> GCTGTGAA AGACACAGAACAGTACAGGGTGT GGTCCCTGCCCTCGAGAGGAT 3'	cloning - mut #10 complementary oligonucleotide
sense WAF1 3'UTR mut #11	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCT <u>TGTG</u> ATGCTGAATAT ACAGCAGGTGCT 3'	cloning - mut #11 complementary oligonucleotide
antisense WAF1 3'UTR mut #11	5' CTAGAGCACCTGCTGTATATTCA GCAT <u>CACA</u> AGAGGAGCTGTGAAAG ACACAGAACAGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #11 complementary oligonucleotide
sense WAF1 3'UTR mut #12	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAGCATCGAATA TACAGCAGGTGCT 3'	cloning - mut #12 complementary oligonucleotide
antisense WAF1 3'UTR mut #12	5' CTAGAGCACCTGCTGTATATTC <u>G</u> <u>ATGCT</u> GTGGGAGGAGCTGTGAAAG ACACAGAACAGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #12 complementary oligonucleotide
sense WAF1 3'UTR mut #13	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTC ACAGCTCCTCCACAATGCT <u>AGGC</u> <u>GTACAG</u> CAGGTGCT 3'	cloning - mut #13 complementary oligonucleotide
antisense WAF1 3'UTR mut #13	5' CTAGAGCACCTGCTGTACGCCTA GCATTGTGGGAGGAGCTGTGAAAG ACACAGAACAGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #13 complementary oligonucleotide
sense WAF1 3'UTR mut #14	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA <u>CGTGAC</u> AGGTGCT 3'	cloning - mut #14 complementary oligonucleotide
antisense WAF1 3'UTR mut #14	5' CTAGAGCACCTGTCACGTATTC AGCATTGTGGGAGGAGCTGTGAA AGACACAGAACAGTACAGGGTG TGGTCCCTGCCCTCGAGAGGAT 3'	cloning - mut #14 complementary oligonucleotide
sense WAF1 3'UTR mut #15	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGTGAACGCT 3'	cloning - mut #15 complementary oligonucleotide
antisense WAF1 3'UTR mut #15	5' CTAGAGCGTTC <u>ACT</u> GTATATTCA GCATTGTGGGAGGAGCTGTGAAAG ACACAGAACAGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #15 complementary oligonucleotide
sense WAF1 3'UTR mut #16	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGCAGGT <u>ATT</u> 3'	cloning - mut #16 complementary oligonucleotide

antisense WAF1 3'UTR mut #16	5' CTAGA <u>A</u> TACCTGCTGTATATTCA GCATTGTGGGAGGAGCTGTGAAAG ACACAGAACAGTACAGGGTGTGGT CCCTGCCCTCGAGAGGAT 3'	cloning - mut #16 complementary oligonucleotide
FWD -210WAF1-CAT poly(A) destroy	5' CCATTATAAGCTGCACTAGTCA AGTTAACAAC 3'	Site - directed mutagenesis primer
REV -210WAF1-CAT poly(A) destroy	5' GTTGTTAACTTGACTAGTGCAGC TTATAATGG 3'	Site - directed mutagenesis primer
sense WAF1 3'UTR wt + poly(A) signal	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA C AGCTCCTCCACAATGCTGAAT ATACAGCAGGTGCTCAATAAATGA TTCTTAGTGACTTTT 3'	cloning - wt + poly (A) signal complementary oligonucleotide
antisense WAF1 3'UTR wt + poly(A) signal	5'CTAGAAAAGTCACTAAGAATCA TTTATTGAGCACCTGCTGTATATTC AGCATTGTGGGAGGAGCTGTGAAA GACACAGAACAGTACAGGGTGTGG TCCCTGCCCTCGAGAGGAT 3'	cloning - wt + poly (A) signal complementary oligonucleotide
sense WAF1 3'UTR mut#15 + poly(A) signal	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGTGAACGCTCAATAAATGAT TCTTAGTGACTTTT 3'	cloning - mut#15 + poly (A) signal complementary oligonucleotide
antisense WAF1 3'UTR mut#15 + poly(A) signal	5' CTAGAAAAGTCACTAAGAATCA TTTATTGAGCGTTCACTGTATATTC AGCATTGTGGGAGGAGCTGTGAAA G ACACAGAACAGTACAGGGTGTG GT C CCTGCCCTCGAGAGGAT 3'	cloning - mut#15 + poly (A) signal complementary oligonucleotide
sense WAF1 3'UTR mut#15/16 + poly(A) signal	5' ATCCTCTCGAGGGCAGGGACCAC ACCCTGTACTGTTCTGTGTCTTTCA CAGCTCCTCCACAATGCTGAATA TACAGTGAACATCTAATAAATGAT TCTTAGTGACTTTT 3'	cloning - mut#15/16 + poly (A) signal complementary oligonucleotide
antisense WAF1 3'UTR mut#15/16 + poly(A) signal	5' CTAGAAAAGTCACTAAGAATCA TTTATTAGATGTTCACTGTATATT CAGCATTGTGGGAGGAGCTGTGAA AGACACAGAACAGTACAGGGTGTG GTCCCTGCCCTCGAGAGGAT 3'	cloning - mut#15/16 + poly (A) signal complementary oligonucleotide

## 3.2. Culture of Cell Lines

### 3.2.1. Culture and Maintenance of Cell Lines

The cell lines utilized in this study were obtained from the American Type Culture Collection (ATCC). The cell culture media used for cell maintenance was purchased by Invitrogen – Gibco Cell Culture Systems. HT29, WiDr, LS 174T, SW480 colorectal adenocarcinoma cells and the SW620 lymph node metastasis derived

colorectal cells were grown in Dulbecco's Modified Eagle's Medium (DMEM). The COLO 201, COLO 205, COLO 320 and DLD-1 colon adenocarcinoma cells were maintained in RPMI-1640 medium. The T-47D breast ductal carcinoma cells were grown in RPMI-1640 media with 0.2 units / mL Humulin. The HepG2 hepatocellular carcinoma cells were grown in Dulbecco's modified Eagle's medium and Ham's F-12 medium. BHK-21 baby hamster kidney cells and the temperature sensitive *tsBN462* cells derived from them were obtained from Dr. T. Sekiguchi (Kyushu University, Fukuoka Japan) and maintained in DMEM. All cells described were grown in media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and grown in a CO<sub>2</sub> Incubator 3326 at 5% CO<sub>2</sub> and 37°C, except *tsBN462* cells which were sustained at 32.6°C and 5% CO<sub>2</sub>. Upon expansion and cell seeding, adherent cells were dislodged from their tissue culture plate using Trypsin-EDTA.

### **3.2.2. Histone Deacetylase Inhibitor and Cycloheximide Treatments**

Cells were trypsinized, seeded and grown to approximately 50% confluency. Five mM sodium butyrate (NaB), or 1.0 µM Trichostatin A (TSA) was added directly to the growth media and treated cells were harvested at subsequent time intervals. In dose response experiments, cells were treated with 0.5, 1.0, 2.0, 5.0 and 10.0 mM NaB and harvested 24 hours following exposure. In a similar manner, tumor cells were propagated to 50% confluency and treated with 50 µM cycloheximide (CHX) alone or in unison with 5.0 mM NaB or 1.0 µM TSA, added directly to the cell culture media.

### **3.2.3. Actinomycin D Treatments**

Cells were grown to 50% confluency as described above and treated with 5.0 µg/mL actinomycin D (Act.D), added directly to the growth media for mRNA half-life studies. Alternatively, cells were pre-treated 12 hours with 5.0 mM NaB or 1.0 µM TSA prior to the addition of 5.0 µg/mL Actinomycin D.

### **3.3. Cellular Growth Curve Experiments**

Confluent SW480, HT29, T-47D and HepG2 cells were trypsinized, seeded at  $1.0 \times 10^5$  cells / 35 mm well (9.6cm<sup>2</sup>), and allowed to grow under normal conditions for

a period of 48 hours. At this time, fresh cell culture media was administered to the cells, which were treated with different doses of NaB (0.5, 1.0, 2.0, 5.0, 10.0 mM), TSA (0.5, 1.0, 2.0, 5.0, 10.0  $\mu$ M), or left untreated. Subsequently, these cells were trypsinized at 24, 48, 72 and 96 hours following treatment and counted in phosphate-buffered saline (PBS, 8 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 1.44 g disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ), and 0.24 g Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) / L of water) with a Coulter Counter ZM .

### **3.4. Bacterial Strains**

The DH5 $\alpha$  and TOP10 competent *Escherichia coli* strains were routinely used as host cells for ligation reactions, site directed mutagenesis, DNA plasmid amplification and glycerol stock plasmid storage in these studies. Individual DH5 $\alpha$  or TOP10 transformed colonies were isolated from Luria-Bertani (LB) plates containing ampicillin, following overnight growth in a 37°C Isotemp Incubator 230D. LB plates were made by combining 2.5% (w/v) LB Broth, Miller with Bacto Agar and water prior to autoclaving. Ampicillin was added to the autoclaved LB agar mixture upon reaching 65°C and LB ampicillin plates were poured and cooled to room temperature. DH5 $\alpha$  and TOP10 transformed plasmids were amplified for plasmid preparations and glycerol stock generation by inoculating LB ampicillin media (lacking Bacto Agar) with the isolated colonies from LB ampicillin agar plates, and grown overnight at 37°C in an ORBIT Incubator Shaker. Plasmid glycerol stocks contained 850  $\mu$ L of overnight cell culture and 150  $\mu$ L 50% glycerol, which were stored at -80°C.

### **3.5. General Molecular Biology Techniques**

The basis of numerous molecular biology techniques described in this thesis have been previously published in a Molecular Cloning Laboratory Manual (Sambrook *et al.*, 1989).

### **3.5.1. Cloning Techniques**

#### **3.5.1.1. Restriction Enzyme Digestion**

Restriction enzyme digests were performed on double stranded DNA in the presence of the recommended buffer and 1 unit of restriction endonuclease /  $\mu\text{g}$  DNA. Reactions proceeded at 37°C for a minimum of 1 hour.

#### **3.5.1.2. Preparation of Blunt End DNA Fragments**

5'-overhangs generated from restriction enzyme digests were filled in by extending 3' recessed ends with DNA polymerase I Klenow Fragment. Twenty five  $\mu\text{L}$  of restriction digested DNA were incubated with 5  $\mu\text{L}$  10X reaction buffer, 0.5  $\mu\text{L}$  5mM dNTP mixture and 5 units DNA polymerase I Klenow fragment. Reactions were incubated at 37°C for 10 minutes and stopped by heating to 70°C for 10 minutes. 3'-overhangs were removed using T4 DNA polymerase. Twenty five  $\mu\text{L}$  of digested DNA was combined with 5  $\mu\text{L}$  of 10X reaction buffer, 0.5  $\mu\text{L}$  5 mM dNTP mixture and 5 units T4 DNA polymerase. Reactions occurred at 15°C for 15 minutes and were terminated by heating to 70°C for 10 minutes.

#### **3.5.1.3. Removal of the 5' Phosphate Group of DNA**

The phosphate group was removed from the DNA 5' termini with either calf intestinal alkaline phosphatase, or shrimp alkaline phosphatase. Calf intestinal alkaline phosphatase was added directly to the DNA at 0.5 units /  $\mu\text{g}$  of DNA to be dephosphorylated. The reaction was carried out at room temperature for 1.5 hours. Conversely, 40  $\mu\text{L}$  of DNA solution was combined with 10 X shrimp alkaline phosphatase reaction buffer and 1 unit of shrimp alkaline phosphatase. This reaction was incubated at 37°C for 30 minutes and stopped by heating at 65°C for 15 minutes.

#### **3.5.1.4. Polymerase Chain Reaction**

Sequence specific, forward and reverse, primers were designed to amplify DNA fragments of interest. In some cases, restriction endonuclease sites were incorporated into the primers to aid in subsequent cloning procedures. DNA fragments were amplified from plasmid DNA vectors using *Taq* DNA polymerase. Alternatively, *Pfu* DNA polymerase was employed to amplify DNA fragments from genomic DNA. The DNA polymerase specific buffer, dNTPs, sequence specific primers, and template DNA were combined prior to DNA polymerase addition. The polymerase chain reaction



(PCR) was performed in a GeneAmp PCR System 2700. Initially, template DNA was denatured at 95°C for 5 minutes, followed by 25 – 35 cycles of: denaturation at 95°C, primer annealing, and primer extension at 72°C or 68°C for *Taq* and *Pfu* DNA polymerase, respectively. Following cycling, a final extension step was performed at the DNA polymerase specified temperature. The optimal primer annealing temperature for each reaction was determined by entering the template and primer sequences into MacVector 7.2.3 software. In addition, the extension time was determined based on the rate of *Taq* extension at 2 kb / minute, and *Pfu* at 0.5 kb / minute.

#### **3.5.1.5. Annealing of Complementary Oligonucleotides**

1 µg of each complementary oligonucleotide was combined in the presence of 10X Annealing Buffer (100 mM Tris (hydroxymethyl) aminomethane hydrochloric acid (HCl) pH 8.0; 1 M NaCl; and 10 mM ethylenediaminetetraacetic acid (EDTA) and heated at 100°C for 5 minutes. The reactions were gradually cooled to room temperature to anneal the complementary oligonucleotides.

#### **3.5.1.6. Agarose Gel Electrophoresis**

DNA gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 30% (v/v) glycerol was added to DNA samples prior to separation on an agarose gel. Agarose gels of 1.0 to 1.5% (w/v) were prepared in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) or TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0), and gel electrophoresis was performed in a Horizontal Gel Electrophoresis System with the corresponding buffer at 100V. Upon completion of electrophoresis, double stranded DNA was stained with 0.5 µg/mL ethidium bromide and DNA fragments were visualized using a Gel Doc 2000 Gel Documentation System.

#### **3.5.1.7. Gel Extraction of Linear DNA Fragments**

Ethidium bromide stained DNA fragments were excised from agarose gels with a scalpel and stored at -20°C. A QIAquick Gel Extraction Kit was used to extract and purify the fragment of DNA as per the manufacture's protocol, with the exception that the samples were not incubated at 50°C, but remained at room temperature and were vortexed to dissolve the gel slice.

#### **3.5.1.8. DNA Ligation Reaction**

A Quick Ligation Kit was employed for DNA ligation reactions. Purified DNA insert and 50 ng of restriction digested, dephosphorylated, purified DNA vector were combined with 10  $\mu$ L of 2 X Quick Ligation Reaction Buffer and 1  $\mu$ L Quick T4 DNA ligase. The reactions were allowed to proceed at room temperature for 10 minutes, placed on ice and stored at -20°C. For vector re-circularization ligations, 100 ng of DNA was used in the ligation reaction.

#### **3.5.2. QuikChange Site-Directed Mutagenesis**

The QuikChange Site-Directed Mutagenesis protocol was adapted from Stratagene. Complementary oligonucleotides were designed between 25 – 45 bases in length, containing unmodified bases flanking a central region of mutated sequence. 10 X *Pfu* DNA polymerase buffer, dNTPs, complementary oligonucleotides, template DNA and *Pfu* DNA polymerase were pooled for amplification. PCR reactions were denatured at 95°C for 5 minutes and underwent 16 cycles of: 95°C denaturation, primer annealing, and extension at 68°C. The extension time was determined based on the vector size and the rate of *Pfu* DNA polymerase extension at 0.5 kb / minute. The final PCR extension time was 7 minutes at 68°C. The optimal annealing temperature was determined using MacVector 7.2.3. Upon completion of amplification, the reaction mixtures were treated with restriction endonuclease using 15 units of DpnI at 37°C for 90 minutes, to digest the parental DNA.

#### **3.5.3. Competent Bacterial Cells**

##### **3.5.3.1. Preparation of TOP10 Cells**

A culture of competent *Escherichia coli* TOP10 cells was grown overnight in 5 mL LB media, shaking at 37°C. The entire overnight starter culture was used to inoculate 100 mL of LB, which was grown under identical conditions until the optical density (OD) 550 nm reached approximately 0.4. At this point, TOP10 cells were collected by centrifugation in a Sorvall RT6000D at 2000-3000 rpm and 4°C for 15 minutes. The cell pellet was resuspended in 30 mL RF1 (100 mM rubidium chloride (RbCl), 50 mM manganese chloride tetrahydrate ( $\text{MnCl}_2 - 4\text{H}_2\text{O}$ ), 30 mM potassium

acetate ( $\text{C}_2\text{H}_3\text{O}_2\text{K}$ , BDH), 10 mM calcium chloride dihydrate ( $\text{CaCl}_2 - 2\text{H}_2\text{O}$ ), 15% (w/v) glycerol, and acetic acid to pH 5.8) and incubated on ice for 15 minutes. Cells were collected, as discussed above, and resuspended in 7.2 mL RF2 (10 mM 3-N-morpholinopropanesulfonic acid (MOPS), 10 mM  $\text{RbCl}$ , 75 mM  $\text{CaCl}_2 - 2\text{H}_2\text{O}$ , 75 mM glycerol, and sodium hydroxide (NaOH) adjusted to pH 6.8). Following a second 15 minute incubation on ice, TOP10 cells were aliquotted into 200  $\mu\text{L}$  fractions, flash frozen and stored at  $-80^\circ\text{C}$ .

### **3.5.3.2. Transformation of Competent TOP10 Cells**

Transformation of bacteria with a ligation reaction was performed by adding 5  $\mu\text{L}$  of the ligation reaction to 100  $\mu\text{L}$  of TOP10 cells. The mixture was incubated on ice for 30 minutes, heat shocked at  $42^\circ\text{C}$  for 30 seconds, and returned to ice for 2 minutes. Next, 500  $\mu\text{L}$  of room temperature LB was added to the transformation reaction and the cells were allowed to grow in a shaking incubator at  $37^\circ\text{C}$ . After 1 hour, cells were spread on LB ampicillin plates, and positioned upside down at  $37^\circ\text{C}$  overnight. For the transformation of intact plasmids, 1  $\mu\text{L}$  of plasmid was added directly to 100  $\mu\text{L}$  of TOP10 cells, which were placed on ice for 5 minutes and plated directly onto LB ampicillin plates.

### **3.5.4. Zero Blunt TOPO PCR Cloning and One Shot TOP10 Cell Transformation**

Blunt end PCR products, generated by *Pfu* DNA polymerase, were inserted into a pCR-Blunt II-TOPO plasmid vector as described in the Zero Blunt TOPO PCR Cloning Kit manual. Briefly, 4  $\mu\text{L}$  of PCR product was combined with a dilute salt solution and the pCR-Blunt II-TOPO plasmid and incubated at room temperature for 5 minutes prior to transformation. Two  $\mu\text{L}$  of the TOPO cloning reaction were transformed into One Shot TOP10 chemically competent *E. coli*. The transformation was incubated on ice for 30 minutes, heat shocked at  $42^\circ\text{C}$  for 30 seconds, and returned to ice. 250  $\mu\text{L}$  of S.O.C. media provided by the manufacturer was added to the transformation reaction, which was subsequently transferred to a shaking  $37^\circ\text{C}$  incubator for 1 hour and spread onto LB plates containing 50  $\mu\text{g} / \text{mL}$  kanamycin.

### **3.5.5. Sequencing**

Automated DNA sequencing to verify cloned genes, PCR products, or newly acquired DNA plasmids was performed by Annette Kerviche at the Saskatchewan Cancer Agency, Health Research Unit using an ABI Prism 310 Genetic Analyser or at the National Research Council Canada, Plant Biotechnology Institute using a 96-capillary AB 3730x/DNA sequence analyser.

### **3.5.6. Plasmid Isolation from *E. coli* Strains**

#### **3.5.6.1. Small-Scale Plasmid Isolation**

An isolated transformed colony from LB selective agar plates was grown overnight in 4 mL of LB ampicillin at 37°C in a shaking incubator. Two mL of the overnight culture was centrifuged in a tabletop Biofuge 13 microcentrifuge at 13,000 rpm to collect the cells for small-scale plasmid isolation. The QIAprep Miniprep or the FastPlasmid Mini Kit was used as per the manufacturer's recommendations for plasmid DNA isolation. The concentration of plasmid DNA was determined by assessing the absorbance at a wavelength of 260 nm with a SmartSpec 3000 Spectrophotometer, and by agarose gel electrophoresis comparative quantitative analysis.

#### **3.5.6.2. Mid-Scale Endofree Plasmid Isolation**

A single transformed colony from a streaked LB ampicillin agar plate was used to inoculate 4 mL of LB ampicillin, which was then transferred to a 37°C shaking incubator. Following approximately 8 hours of incubation the plasmid DNA starter culture was diluted 1:500 or 1:1000 in 100 mL LB ampicillin, for low and high copy number plasmids, respectively, and the diluted culture was grown at 37°C overnight. Transformed cells were collected in a Beckman J2-MI highspeed centrifuge with a JA-10 rotor at 6000 x g for 15 minutes at 4°C. Plasmid DNA purification was performed with a QIAfilter Plasmid Midi Kit, as suggested in the user manual, with a few modifications to ensure that plasmid DNA remained endotoxin-free. Modifications included adding 1.0 mL of Buffer ER to the filter lysate, followed by a 30 minute incubation on ice. Furthermore, DNA was eluted with Buffer QN rather than QF, endotoxin-free 70% ethanol was used to wash the DNA pellet and DNA was eluted in

endotoxin-free Buffer TE. The yield of plasmid DNA was determined as discussed above.

#### **3.5.6.3. Endofree Isolation of Large Scale Plasmid Preparations**

Plasmid DNA starter cultures were initiated as explained above, although starter cultures were diluted in 250 mL of LB ampicillin. Large scale plasmid isolation was carried out with an EndoFree Plasmid Maxi Kit, exactly as recommended by the manufacturer.

#### **3.5.7. DNA Ladder Markers**

DNA size markers were generated by restriction endonuclease digestion of Lambda DNA with EcoRI, or pBluescript KS + DNA with HpaII. Alternatively, an O'GeneRuler 1 kb DNA Ladder or an O'GeneRuler 50 bp DNA Ladder was utilized.

### **3.6. Information on Plasmids and Construction Details**

#### **3.6.1. Expression Vectors and cDNAs**

The c-Src cDNA expression vector, Y530 wt c-Src pcDNA3.1 was obtained from Dr. Keith Bonham. Human c-Src Family Kinase (SFK) Expressed Sequence Tags (ESTs) corresponding to the mRNA sequence of Lck (GenBank Accession Number BG756533), Yes (BI560649), Fyn (BM920742) and Lyn (BE676457) were obtained from ATCC (ATCC Numbers: 6883710, 7497767, 7997662, and 5509761 respectively). The HHCI89 clone, which contains a 1.1 kb  $\beta$ -actin cDNA insert in a pBluescript SK- vector was obtained from ATCC. The pCMV  $\beta$ -Gal expression vector was acquired from Dr. W. Roesler (University of Saskatchewan). pCMV hTAF<sub>II</sub>250 was a gift from Dr. R. Tjian (Howard Hughes Medical Institute, University of California at Berkeley) and contains a 6 kb wt TAF<sub>II</sub>250 cDNA insert (Wang *et al.*, 1994). The TAF1 acetyltransferase (AT) mutants: pCS2+ HA-TAF1  $\Delta$ 848-850 ( $\Delta$ Stu), pCS2+ HA-TAF1  $\Delta$ 844-850 ( $\Delta$ MED), pCS2+ HA-TAF1  $\Delta$ 574-590, and pCS2+ HA-TAF1 G923/925D double mutant were acquired from Dr. E. Wang (University of Washington) and have been previously documented (Hilton *et al.*, 2005). The p21WAF1 expression vector, pCEP-WAF1, was obtained from Dr. B. Vogelstein

(Johns Hopkins Oncology Center) and contains a 2.1 kb p21WAF1 cDNA fragment including the 5' untranslated, coding and 3' untranslated regions (el-Deiry *et al.*, 1993).

### **3.6.2. c-Src Family Kinase CAT Reporter Constructs**

The 0.38SRC1A-chloramphenicol acetyltransferase (CAT) vector contains the pGEM2-CAT reporter vector backbone and has been previously described (Bonham and Fujita, 1993). The pCAT3-Basic backbone was used in the construction of the -145SRC1 $\alpha$  reporter vector and has also been described (Bonham *et al.*, 2000). pYSCAT2 was obtained from Dr. T. Yamamoto (University of Tokyo) and contains a 2.1kb YES promoter fragment (Matsuzawa *et al.*, 1991). The -630LCK-CAT promoter construct was designed by amplifying an 808 bp fragment encompassing the LCK proximal promoter from Colo 205 genomic DNA (Takadera *et al.*, 1989). A sense primer (Fwd LCK: 5' CATGGTACCTTAAACTTTGAGGACAGCGCTTTCC 3' introduced a 5' KpnI restriction site, and an antisense primer (REV LCK: 5' CTCCTCGAGATCTGCTC-CCCCAACATCAG 3' introduced a XhoI site at the 3' end of the -630LCK promoter fragment. The -630LCK-CAT reporter construct was generated by digesting both pCAT3-Basic and the -630LCK PCR fragment with KpnI and XhoI, followed by ligation of the -630LCK fragment into the pCAT3-Basic vector. The -590LYN-CAT construct was constructed in a similar fashion by amplifying a 631 bp fragment, encompassing the LYN promoter, from COLO 205 genomic DNA (Uchiumi *et al.*, 1992). A sense primer (FWD LYN -593): 5' CCCGGTACCGAAAGG TACACG 3' was used to introduce a KpnI restriction site to the -590LYN fragment, and an antisense primer (REV LYN +14): 5' CGGCTCGAGAGGAACTGGCTG 3' a XhoI site.

### **3.6.3. p21WAF1 Reporter Constructs**

The pWWP-luciferase (WAF1-Luc) vector was initially obtained from Dr. B. Vogelstein (Howard Hughes Medical Institute, Johns Hopkins Oncology Center) (el-Deiry *et al.*, 1993), and further modified by Scott Dehm to generate the -2300WAF1-CAT, -210WAF-CAT and -101WAF1-CAT reporter vectors (Dehm *et al.*, 2004).

#### **3.6.4. -210WAF1-CAT + p21WAF1 3'UTR Reporter Vectors**

The -210WAF1-CAT reporter construct was digested with SpeI and SacII enzymes, blunt ends were created and the vector re-ligated to remove a 19 bp fragment, containing a XbaI restriction site immediately upstream of the -210WAF1 promoter. This vector, -210WAF1-CAT ΔXbaI, still contained one XbaI restriction site between the CAT coding gene and the SV40 late poly(A) region. The -210WAF1-CAT ΔXbaI vector was used as the parental vector for the majority of the subsequent -210WAF1-CAT + p21WAF1 3'UTR plasmids. To generate the -210WAF1-CAT + p21WAF1 3'UTR (591-2079) vector, the full length p21WAF1 3'UTR (591-2079) was amplified from the pCEP-WAF1 p21WAF1 cDNA expression vector. PCR was performed using *Taq* DNA polymerase, the forward primer (FWD p21WAF1 3'UTR 591): 5' GGAAGTCTAGAGTCCTGGAAG 3', and reverse primer (REV p21WAF1 3'UTR 2079): 5' CGTTTCTAGAGCACCTGCTG 3', each containing built-in XbaI restriction endonuclease sites. The resultant PCR product was restriction digested with XbaI and ligated into a previously XbaI linearized -210WAF1-CAT ΔXbaI vector to create the -210WAF1-CAT + p21WAF1 3'UTR (591-2079) plasmid.

##### **3.6.4.1. -210WAF1-CAT + p21WAF1 3'UTR 5' Deletion Plasmids**

The -210WAF1-CAT + p21WAF1 3'UTR (712-2079) plasmid was constructed as described above for -210WAF1-CAT + p21WAF1 3'UTR (591-2079), but the 1367 bp p21WAF1 3'UTR fragment was amplified with the FWD p21WAF1 3'UTR 712 primer: 5' CATACTCTAGACGCCCCCTGC 3' and the REV p21WAF1 3'UTR 2079 primer. Similarly, p21WAF1 3'UTR (788-2079) and p21WAF1 3'UTR (840-2079) sequences were amplified with the FWD p21WAF1 3'UTR 780 primer: 5' GAGGTTTCTAGAAGTGCTGGGC 3' and the FWD p21WAF1 3'UTR 840 primer: 5' CCTCATCTAGAGTTCTCCTTTTCC 3', respectively with the REV p21WAF1 3'UTR 2079 primer. XbaI digested PCR products were individually ligated with the XbaI cut -210WAF1-CAT ΔXbaI vector to generate: -210WAF1-CAT + p21WAF1 3'UTR (712-2079), -210WAF1-CAT + p21WAF1 3'UTR (788-2079), -210WAF1-CAT + p21WAF1 3'UTR (840-2079) plasmids. The -210WAF1-CAT + p21WAF1 3'UTR (591-840) vector was built in the same fashion by first performing PCR

amplification with the FWD p21WAF1 3'UTR 581 primer and REV p21WAF1 3'UTR 833 primer: 5' GAGAACTCTAGATGAGGAGGC 3'.

#### **3.6.4.2. -210WAF1-CAT + p21WAF1 3'UTR Internal Deletion Plasmids**

Internal deletion constructs -210WAF1 + p21WAF1 3'UTR (932-2079), -210WAF1 + p21WAF1 3'UTR (1045-2079), -210WAF1 + p21WAF1 3'UTR (1173-2079), -210WAF1 + p21WAF1 3'UTR (1272-2079), and -210WAF1 + p21WAF1 3'UTR (840-1470) were created exactly as described above. The REV p21WAF1 3'UTR 2079 primer with the FWD p21WAF1 3'UTR 932: 5' GTACCCTCTAGAGGG GTGTGGCTC 3', FWD p21WAF1 3'UTR 1045: 5' GGCATCTAGAAGGGGCCTCA C 3', FWD p21WAF1 3'UTR 1173: 5' GTACCCTCTAGACTCTTGATACC 3', or FWD p21WAF1 3'UTR 1272: 5' CCTGCTCTAGAGAGCCCGTCTC 3' primer was used to amplified the respective p21WAF1 3'UTR sequences. The FWD p21WAF1 3'UTR 840 primer and a REV p21WAF1 3'UTR 1470 primer: 5' CCCCTTCTAGACC ATTGAGCTGGG 3' were used in the PCR reaction of the p21WAF1 3'UTR 840-1470 region.

#### **3.6.4.3. -210WAF1-CAT + p21WAF1 3'UTR 3' Deletion Plasmids**

PCR amplification was performed to establish four different p21WAF1 3'UTR 3' deletions. The FWD p21WAF1 3'UTR 591 primer was used in all four reactions with either the REV p21WAF1 3'UTR 2063: 5' CCTGCTCTAGATTTCAGCATTG 3', REV p21WAF1 3'UTR 2031: 5' GAAAGATCTAGAACAGTACAGG 3', REV p21WAF1 3'UTR 2000: 5' GCCCTCTAGAGGTTTACAGTC 3', or REV p21WAF1 3'UTR 1979 primer: 5' CTAGGTCTAGAAACGGGAACCAGG 3'. The p21WAF1 3'UTR 1467-2079 fragment was amplified with the FWD p21WAF1 3'UTR 1467 primer: 5' CCCCATCTAGATGGACTGGAAGG 3' and REV p21WAF1 3'UTR 2079 primer. The PCR products were treated with restriction endonuclease XbaI and ligated into XbaI linearized -210WAF1-CAT ΔXbaI to create the -210WAF1 + p21WAF1 3'UTR (591-2063), -210WAF1 + p21WAF1 3'UTR (591-2031), -210WAF1 + p21WAF1 3'UTR (591-2000), -210WAF1 + p21WAF1 3'UTR (591-1979), and -210WAF1 + p21WAF1 3'UTR (1467-2079) plasmids.



#### **3.6.4.4. -210WAF1-CAT + p21WAF1 3'UTR Mutant Constructs**

The -210WAF1-CAT + p21WAF1 3'UTR (591-2079) plasmid vector was altered via two mutagenesis reactions. First, the upstream XbaI restriction site between the CAT coding gene and p21WAF1 3'UTR was abolished using the complimentary sense (FWD -210WAF1 XbaI destroy): 5'GCGGGGCGTAATCTGCAGTCCTGGAA-GCGCG 3' and antisense (REV -210WAF1 XbaI destroy): 5' CGCGCTTCCAGGAC-TGCAGATTACGCCCCGC 3' primers, to create -210WAF1-CAT + p21WAF1 3'UTR (591-2079) -XbaI. Second, an EcoRV restriction endonuclease site was created in the -210WAF1-CAT + p21WAF1 3'UTR (591-2079) -XbaI vector at position 1991 of the p21WAF1 3'UTR with the respective primers, FWD p21WAF1 3'UTR EcoRV create: 5' CTCCACCTAGACTGATATCCTCTCGAGGGCAGG 3' and REV p21WAF1 3'UTR EcoRV create: 5' CCTGCCCTCGAGAGGATATCAGTCTAGGTGGAG 3'. The vector generated from the above two mutagenesis reactions was named -210WAF1-CAT + p21WAF1 3'UTR (591-2079) -XbaI / +EcoRV, and was used as the parental vector for the construction of the -210WAF1 + p21WAF1 3'UTR mutant vectors. Sixteen separate -210WAF1 + p21WAF1 3'UTR mutant constructs (mut #1 - 16) were created. Complementary mutant oligonucleotides were designed that contained the 1997 – 2077 region of the p21WAF1 3'UTR surrounded by EcoRV and XbaI adaptor nucleotides. The oligonucleotide pairs were annealed and individually ligated into an EcoRV / XbaI cut -210WAF1-CAT + p21WAF1 3'UTR (591-2079) -XbaI / +EcoRV vector to generate the mutant vectors, -210WAF1-CAT + p21WAF1 3'UTR mut #1 - #16. Each mutant oligonucleotide pair contained five consecutive base pair mutations, beginning at position 2000 of the p21WAF1 3'UTR, where pyrimidines were replaced with pyrimidines (C to T, or T to C) and purines for purines (A to G, G to A). Mutant # 16 oligonucleotides were the exception, where only two base pairs were mutated at 2076 and 2077 of the p21WAF1 3'UTR. The oligonucleotide pairs utilized in the above cloning experiments are listed in Table 3.5, with the mutated nucleotide sequence underlined.

#### **3.6.4.5. -210WAF1-CAT + p21WAF1 3'UTR + Poly(A) Signal Constructs**

The -210WAF1-CAT + p21WAF1 3'UTR (591-2079) -XbaI / +EcoRV vector SV40 late poly(A) signal was destroyed by QuikChange site directed mutagenesis,

creating a SpeI restriction endonuclease site in its place. Mutagenesis was carried out with the primers, FWD -210WAF1-CAT poly(A) destroy: 5' CCATTATA AGCTGCACTAGTCAAGTTAACAAC 3' and REV -210WAF1-CAT poly(A) destroy: 5' GTTGTTAACTTGACTAGTGCAGCTTATAATGG 3' to generate -210WAF1-CAT + p21WAF1 3'UTR (591-2079) –XbaI / +EcoRV / Δpoly(A) signal. New wt, mutant #15 and mutant #15/16 p21WAF1 3'UTR + poly(A) signal oligonucleotides were purchased from IDT that spanned 1997 – 2102 and contained the p21WAF1 3'UTR poly(A) signal (Table 3.5, mutated sequences are underlined and the poly(A) signals are identified in bold). Similar to the oligonucleotides discussed above, EcoRV and XbaI adaptor nucleotides were placed at the termini of these oligonucleotides for cloning purposes. The complementary oligonucleotides were annealed and ligated into an EcoRV / XbaI cut -210WAF1-CAT + p21WAF1 3'UTR (581-2079) –XbaI / +EcoRV / Δpoly(A) signal plasmid. The positive clones were digested with XbaI / SpeI, to remove a 145 bp region encompassing the upstream SV40 late poly(A) region leading up to the late SV40 destroyed poly(A) signal. The vector backbone was ligated to re-circularize and create the -210WAF1-CAT + p21WAF1 3'UTR wt poly(A) signal, -210WAF1-CAT + p21WAF1 3'UTR mut#15 poly(A) signal, and -210WAF1-CAT + p21WAF1 3'UTR mut#15/16 poly(A) signal plasmids.

### **3.7. Transient Transfections of Cultured Cells**

#### **3.7.1. Standard Transfection Conditions**

Confluent SW480, HepG2, HT29 cells were counted and seeded at  $5.0 \times 10^5$ ,  $2.5 \times 10^5$ , and  $3.5 \times 10^5$  cells / 35 mm tissue culture plate, respectively. Following seeding, cells were allowed to proliferate under normal growth conditions for 24 hours prior to transient transfection. A standard transfection mixture contained: 1.0 µg of CAT reporter plasmid, 0.5 µg of CMV β-Gal, 0.5 µg pBlue, 85 µL of serum free DMEM media and 10 µL of the SuperFect Transfection Reagent. The mixture was incubated at room temperature for 20 minutes, and the cells were prepared for transfection by washing with PBS. After 20 minutes, 600 µL of DMEM containing 10% FBS was added to the transfection mixture. This transfection mix was then administered to the cells at 670 µL / 35 mm tissue culture plate and the cells were returned to 5% CO<sub>2</sub> and

37°C. Three hours later, the cell transfection was supplemented with 2 mL of the cell type specific media containing 10% FBS. The transfected cells were incubated under standard growth conditions for 45 more hours prior to harvesting.

p21WAF1 transfections were carried out in a similar manner with 0.5 µg of p21WAF1 promoter CAT construct, 0.5 µg CMV β-Gal, 1.0 µg of pBlue and 85 µL of serum free DMEM mixed together with 10 µL of SuperFect and incubated for 20 min. at room temperature. The transfection mix was diluted further with 600 µL DMEM containing 10% FCS and added to directly to HT29 or HepG2 cells seeded the previous day. Likewise, co-transfection mixtures were prepared containing 1.0 µg of WAF1 promoter CAT construct, 1.0 µg CMV β-Gal, 1.0 µg of pBlue and 1.0 µg of CMV c-jun (ATTC). The plasmid preparations used in all transient transfections were endotoxin-free, prepared with either a QIAfilter Plasmid Midi Kit or an EndoFree Plasmid Maxi Kit.

### **3.7.2. Histone Deacetylase Inhibitor Transfection Procedure**

Cells were transfected as described under standard transfection conditions, however 24 hours following the transfection, cell media was removed and replaced with either 2.5 mL of fresh cell culture media (untreated), media containing 5.0 mM of NaB or 1.0 µM of TSA. Following treatment, cells were grown under normal conditions for a 6, 12, or 24 hour time periods before harvesting. Untreated transfected cells were harvested 24 hours following media replacement.

### **3.7.3. Transfection of BHK-21 and tsBN462 Cells**

#### **3.7.3.1. BHK-21 and tsBN462 Transfections and Histone Deacetylase Inhibitors**

BHK-21 and tsBN462 confluent cells were counted and seeded at  $2.5 \times 10^5$  cells / 35 mm tissue culture plate and placed at 32.6°C and 5% CO<sub>2</sub> for 24 hours. One half µg of CAT reporter construct, 0.5 µg of CMV β-Gal, 1.0 µg of pBluescript, 85 µL of serum free DMEM media and 10 µL of the SuperFect Transfection Reagent were pooled to generate the transfection mixture. This mix was incubated at room temperature for 20 minutes and the cells were washed with PBS. 600 µL of DMEM

plus serum was added to the transfection components, and 670  $\mu$ L of the diluted mixture was directly dispensed to the cells. The cells were returned to 32.6°C and 5% CO<sub>2</sub> growth conditions. Following three hours, the transfection mix was removed from the cell tissue culture plate, the cells were washed with PBS, and fresh DMEM containing 10% FBS was added. The cells were allowed to proliferate at 32.6°C for an additional 21 hours, at which time their media was withdrawn again and replaced with 5.0 mM NaB or 1.0  $\mu$ M TSA treated media or fresh DMEM. The BHK-21 and *tsBN462* cells were either transferred to 39.5°C, their restrictive temperature, or maintained at 32.6°C. All cells were harvested 24 hours later.

### **3.7.3.2. *tsBN462* Co-transfections**

*tsBN462* cells were seeded as discussed above. Co-transfections were performed with the c-Src family kinase (SFK) CAT reporter plasmids and TAF1 or TAF1 acetyltransferase (AT) mutants. The transfection mix was comprised of 0.5  $\mu$ g of the CAT reporter construct, 0.25  $\mu$ g of TAF1 (or mutant TAF1), 0.5  $\mu$ g CMV  $\beta$ -Gal, 0.75  $\mu$ g pBluescript, 85  $\mu$ L of serum free DMEM media and 10  $\mu$ L of the SuperFect Transfection Reagent. When TAF1 was not included in the transfection mix, an additional 0.25  $\mu$ g of pBluescript was included. The *tsBN462* cells were transfected with this mixture as discussed above and placed at 32.6°C or 39.5°C 24 hours following transfection initiation, however the cell culture media was not replenished at this time. Cells were harvested 24 hours after the temperature shift occurred.

## **3.8. Determination of CAT Reporter Activity**

### **3.8.1. Chloramphenicol AcetylTransferase Assay**

Chloramphenicol acetyltransferase (CAT) assays were performed on transiently transfected cells with a CAT Colorimetric Enzyme-Linked Immunosorbent Assay Kit, based on the instruction manual. Transiently transfected cells in 35 mm tissue culture plates were washed with PBS and harvested in 500  $\mu$ L of 1 X CAT lysis buffer. Two hundred  $\mu$ L of each lysed cell sample was added to an anti-CAT coated microplate well and samples were incubated at 37°C for a minimum of 1 hour. The plates were subsequently washed five times with 1 X Washing Buffer. 200  $\mu$ L of 1:100 digoxigenin labeled CAT antibody (Anti-CAT-DIG), diluted in Sample Buffer was aliquoted to the

wells, and the microplates were returned to 37°C. Following at least 1 hour at 37°C, the microplates were subject to five washes with 1 X Washing Buffer and 200 µL of an antibody DIG conjugated to peroxidase (Anti-DIG-POD), diluted 1:133 in Sample Buffer, was added to the wells. Samples were once again placed at 37°C for an hour, followed by a final set of five washes. Two hundred µL of POD substrate, without enhancer, was added to the wells and the color was allowed to develop at room temperature. The absorbance of the CAT samples was measured using a Microplate Reader Model 3550, at a measurement wavelength of 405 nm and reference wavelength of 490 nm.

### **3.8.2. Beta-Galactosidase Assay**

β-Galactosidase activity was monitored by adding 30 µL of 1 X CAT lysis buffer harvested transfected cell sample to 85.5 µL of 0.1M sodium phosphate (41 mL 0.2 M Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O, 9 mL sodium dihydrogen phosphate (NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O), 50 mL H<sub>2</sub>O), 1.5 µL of 100 X Mg<sup>2+</sup> solution (0.1 M MgCl<sub>2</sub>, 4.5 M β-mercaptoethanol), and 33 µL of o-nitrophenyl-β-D-galactopyranoside (ONPG) (Hall *et al.*, 1983). The reaction mixtures were placed at 37°C until a yellow color developed, and color development was halted by the addition of 120 µL of 1 M sodium carbonate (NaCO<sub>3</sub>) Stop solution. The sample absorbance, following the addition of Stop solution, was measured with a Microplate Reader at a wavelength of 415 nm.

### **3.8.3. Bradford Protein Assay**

Protein concentration analysis of transiently transfected cells was performed with a Bio-Rad Protein Assay Dye Reagent Concentrate diluted 1:10 in water. 15 µL of 1 X CAT lysis buffer harvested cell sample was added directly to the diluted Protein Assay Dye Reagent and the absorbance, at 595 nm, was determined with a Microplate Reader.

### 3.9. RNA Isolation from Cultured Cells

Total RNA was isolated from cells based on the guanidinium thiocyanate method (Chomczynski *et al.*, 1987). Cells were lysed and harvested in 500  $\mu$ L of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ), 0.5% (%w/v) N-lauroyl sarcosine). Fifty  $\mu$ L of 2 M sodium acetate ( $\text{CH}_3\text{COONa}$ ) pH 4.0, 500  $\mu$ L of water saturated phenol and 100  $\mu$ L of chloroform:isoamyl alcohol mix (49 mL chloroform:1 mL isoamyl alcohol) were added sequentially to each harvested sample, and mixed thoroughly after each addition. Samples were incubated on ice for 15 minutes and centrifuged at 12,000 x g for 2 minutes. The top, aqueous layer was collected in a fresh microcentrifuge tube and isopropanol was added to make up 75% of the total volume. Samples were placed at  $-20^\circ\text{C}$  for a minimum of 1 hour. Upon removal from the freezer, samples were centrifuged at 12,000 x g for 15 minutes, the liquid was removed and the resultant pellet was resuspended in Solution D including  $\beta$ -mercaptoethanol and 400  $\mu$ L of 95% Ethanol. The RNA preparation was placed on ice until precipitation was apparent, and centrifuged at 12,000 x g for 15 minutes. The RNA pellet was washed once with 70 % ethanol and ultimately resuspended in 0.1 mM EDTA containing 0.1% (v/v) diethyl pyrocarbonate water. RNA concentration was determined by measuring the absorbance at a wavelength of 260 nm, where an  $A_{260}$  reading of 1 = 40  $\mu\text{g/mL}$  RNA. RNA purity was identified by the  $A_{260}/A_{280}$  ratio, where a ratio of 1.6 – 2.1 was considered pure RNA.

### 3.10. Northern Blot

#### 3.10.1. Northern Blot Electrophoresis and Gel Transfer

The RNA denaturing gel was prepared by boiling and dissolving 0.8 g agarose in 8 mL of 10 X morpholinopropanesulfonic acid (MOPS) running buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA) and 72 mL  $\text{H}_2\text{O}$ . After cooling the agarose gel to  $60^\circ\text{C}$ , 6% (v/v) of formaldehyde was added and the gel was poured in an RNAase free gel tray. Samples were prepared by combining the volume equivalent of 15  $\mu\text{g}$  total RNA with 10  $\mu$ L of Northern Juice (50.7 % (v/v) formamide, 11.3 % (v/v) 10 X MOPS, 6.8 % (v/v) formaldehyde, 5.6 % (v/v) of glycerol, in diethyl pyrocarbonate treated  $\text{H}_2\text{O}$  containing 0.1 mM EDTA) and  $\text{H}_2\text{O}$  up to a total volume of

25  $\mu$ L. These samples were heated at 65°C for 5 minutes and cooled on ice for 5 minutes prior to the addition of 6  $\mu$ L of a 6 X DNA gel loading buffer (10  $\mu$ L) / ethidium bromide (10  $\mu$ g) mix. Electrophoresis was performed in 1 x MOPS running buffer and the samples were separated on the RNA denaturing gel at 90 V. Following electrophoresis, the Gel Doc was used to photograph the RNA gel to ensure all the RNA samples had been evenly loaded. The gel was washed in H<sub>2</sub>O, and denatured in 50 mM NaOH for 15 minutes. Subsequently, the gel was rinsed with H<sub>2</sub>O, neutralized in 100 mM Tris pH 8.0 for 15 minutes, and a final wash was carried out for 15 minutes in H<sub>2</sub>O. The RNA gel was then transferred overnight in 50 mM sodium phosphate solution, pH 7.0 onto a GeneScreen Plus Hybridization Transfer Membrane. Following gel transfer, the membrane was washed with 1 X SSC (150 mM NaCl, 15 mM sodium citrate) and auto-crosslinked in a UV Stratalinker 2400.

### **3.10.2. Northern Probes**

Northern probes were generated by endonuclease restriction digestions, followed by gel extraction of the appropriately fractioned DNA fragment using the QIAquick Gel Extraction Kit. More specifically, the HHCI89 clone was treated with EcoRI to liberate a 1.1 kb  $\beta$ -actin cDNA fragment. KpnI and NcoI were used to isolate the 876 bp c-Src probe from the Src (wt) Y530 pcDNA 3.1 plasmid. A 500 bp cDNA probe specific for Lck was generated by digesting the Lck EST with RsrII and NcoI. Similarly SacI and HindIII were used to restrict a 523 bp cDNA fragment from the Lyn EST. The Fyn EST was digested with KpnI to produce a 431bp probe, and c-Yes was treated with the BamHI and HincII restriction endonucleases to create a 560 bp cDNA. A 500 bp c-myc probe was generated by PstI digestion of the c-myc cDNA vector, pMI. p21WAF1 plasmid cDNA pCMV-Cip1 was obtained from ATCC and was digested with EcoRI and XhoI to liberate a 1 kb cDNA fragment. The ribosomal protein (RPPO) cDNA was obtained from Dr. W. Roesler (University of Saskatchewan). All cDNA probes were [ $\alpha$ -<sup>32</sup>P] dCTP labeled with either the RediPrime II DNA Labeling System (GE Healthcare) or Ready-To-Go DNA Labeling Beads (-dCTP) as per manufacturer instructions. [ $\alpha$ -<sup>32</sup>P] labeled probe reactions were further purified using a QIAquick Nucleotide Removal Kit to remove unincorporated radiolabeled nucleotides.

### **3.10.3. Northern Blot Hybridization**

RNA crosslinked membranes were prehybridized in a hybridization tube with ExpressHyb Hybridization Solution for a minimum of 1 hour at 65°C in a Micro Hybridization Incubator Model 2000. [ $\alpha$ -<sup>32</sup>P] dCTP-labeled cDNA probes of interest were added directly to the ExpressHyb solution in the tube and the hybridization reaction allowed to proceed overnight at 65°C. Subsequently, hybridized membranes were put through a series of 20 minute washes at 65°C in Wash Buffer 1 (0.3 M NaCl, 30 mM sodium citrate, 1% dodecyl sodium sulfate (SDS), Wash Buffer 2 (75 mM NaCl, 7.5 mM sodium citrate, 1% SDS) and Wash Buffer 3 (30 mM NaCl, 3 mM sodium citrate, 1% SDS). Hybridization signals were acquired with the use of a Molecular Imager FX following exposure to an Imaging Screen K (Bio-Rad Laboratories) or by autoradiography at -80°C on BioMax XAR film with the aid of an intensifier screen. The Molecular Imager was used to quantify RNA signals in some instances.

## **3.11. Western Blot**

### **3.11.1. Lowry Protein Assay**

Cells were harvested in a SDS sample buffer containing 10% (v/v) glycerol, 5% (v/v)  $\beta$ -mercaptoethanol, 2% (w/v) SDS, 65 mM Tris-HCl (pH 7.0), and 0.05% (w/v) bromophenol blue adjusted to pH 6.8. Lowry assays were performed using a Total Protein Kit, Micro Lowry to determine the protein concentration of the samples.

### **3.11.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

A 10% SDS-polyacrylamide resolving gel was prepared containing 10% acrylamide:bis acrylamide (29.2%:0.8%), 375 mM Tris-HCl pH 8.8, 0.1% (v/v) SDS, 0.1% (w/v) ammonium persulfate and 0.04 % (w/v) N,N,N',N'-tetramethylethylenediamine (TEMED) (Sambrook *et al.*, 1989). The 10% acrylamide resolving gel was poured into the Mini-PROTEAN II gel apparatus and layered with water saturated butanol to prevent the gel from warping. Following polymerization, the butanol was removed and a 5% acrylamide stacking gel (29.2% acrylamide:0.8% bis



acrylamide), 130 mM Tris-HCl, 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulfate and 0.04 % (w/v) TEMED) was added above the 10% acrylamide gel (Sambrook *et al.*, 1989). Commonly, 30 µg protein samples were resolved by SDS-PAGE in SDS gel running buffer (25 mM Tris-HCl, 200 mM glycine, 0.1% (w/v) SDS). Prior to loading, all protein samples were boiled for 2 minutes. Electrophoresis was carried out in a Mini-PROTEAN II Cell and allowed to proceed for 50 minutes at 180 volts.

### **3.11.3. Western Blot Method and Antibodies**

Following electrophoresis, the resolved protein was transferred by semi dry electroblotting to Optitran Supported Nitrocellulose and briefly washed in TBST buffer (10 mM Tris, 15 mM NaCl, 0.5% (v/v) TWEEN-20. Subsequently, the membranes were blocked for 1 hour at room temperature in 5% (w/v) fat-free skim milk powder in TBST. Membranes were incubated with the Anti-v-Src (Ab-1) mouse monoclonal antibody at 1 µg/mL or p21WAF1 (187) sc-817 mouse monoclonal antibody at a final concentration of 200 ng/mL in blocking solution. The membrane was washed and incubated for a second time with goat anti-mouse IgG horseradish peroxidase conjugated antibody at 100 ng/mL, which was diluted 1:2000 in blocking buffer. A second wash was performed prior to blot treatment with Western Lightening Chemiluminescence Reagent Plus and exposure to X-OMAT Blue XB-1 film for detection.

### **3.12. Isolation of Nuclear and Cytoplasmic Cellular Extracts**

Cells were harvested in PBS containing 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 1 X Protease Inhibitor Mix (PIM), 0.5 mM dithiothreitol (DTT) and collected by centrifugation at 450 x g at 4°C for 5 minutes. The cell pellet was resuspended in low salt cold Buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.9 with potassium hydroxide (KOH), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 X PIM) and incubated for 10 to 15 minutes on ice to allow the cells to swell. 10% Nonidet-P40 (NP-40) was added to each sample, which was vortexed and subsequently placed on ice for 2 to 5 minutes. Trypan blue was used to assess when the cell membrane had been lysed. The samples were centrifuged at 2500 x

g for 4 minute at 4°C. The resultant supernatant consisting of cytoplasmic extract was saved and the nuclei containing pellet was resuspended in cold hypertonic Buffer C (10 mM HEPES KOH pH 7.9, 25% glycerol, 15 mM MgCl<sub>2</sub>, 420 mM NaCl, 0.5 mM DTT, 0.2 mM PMSF, 1 X PIM) and placed on ice for 20 minutes. The samples were vortexed briefly, centrifuged at 12, 000 x g for 10 minutes to attain the nuclear extracts from the supernatant fraction and snap-frozen. The saved cytoplasmic supernatant was freeze-thawed five times, centrifuged one final time for 10 minutes at 2500 x g, 4°C, aliquoted and snap-frozen (Wang *et al.*, 2000).

### **3.13. RNA Mobility Shift Assays (RMSAs)**

#### **3.13.1. Preparation of the T7 Transcript**

The template pCEP-WAF1 was used to amplify the three sections of the p21WAF1 3' UTR, 586-840, 835-1470, 1459-2079. The extreme 5' of each of the forward primers possessed the T7 promoter sequence 5' CCAAGCTTCTAATACGAC TCACTATAGGGAGA (T<sub>7</sub>). The p21WAF1 3'UTR (T<sub>7</sub>)586-840 sequence was generated with the FWD p21WAF1 3'UTR (T<sub>7</sub>)586 primer: 5' (T<sub>7</sub>) 5' CCTGCAGTCC TGGAAGCGCGAGG 3' and REV p21WAF1 3'UTR 840 primer: 5' GAGAACTCTAG ATGAGGAGGC 3'. The (T<sub>7</sub>)835-1470 region of the 3'UTR was amplified with the FWD p21WAF1 3'UTR (T<sub>7</sub>)835 5' (T<sub>7</sub>) CCCGTGTTCTCCTTTTCCTCTC 3' and REV p21WAF1 3'UTR 1470 5' CCCCTTCTAGACCATTGAGCTGGG 3' primers. Similarly the (T<sub>7</sub>)1459-2079 fragment was amplified using the FWD p21WAF1 3'UTR (T<sub>7</sub>)1459 5' (T<sub>7</sub>) CCAGCTCAATGGACTGGAAGGG 3' and REV p21WAF1 3'UTR 2079 5' CGTTTCTAGAGCACCTGCTG 3' primers.

#### **3.13.2. T7 Reaction and RNA Probe Isolation**

RNA fragments corresponding to ribonucleotides 586-840, 840-1481 and 1467-2079 of the p21WAF1 3' UTR were synthesized by T7 polymerase using the Riboprobe *in vitro* Transcription System Kit as suggested by the manufacturer standard transcription protocol. Following *in vitro* transcription, the reactions were treated with RQ1 RNase-free DNase to remove the template DNA for 15 minutes at 37°C.

Afterward, [ $\alpha$ - $^{32}$ P] rUTP-labeled RNA was extracted from the reactions using an RNeasy Mini Kit.

### **3.13.3. RNA Mobility Shift Assay Method**

Binding reactions between the p21WAF1 3'UTR ribonucleotide sequences and cellular extracts were carried out with Binding Buffer (15 mM HEPES pH 7.9, 10 mM KCl, 10% glycerol, 0.2 mM DTT, 5 mM MgCl<sub>2</sub>), 1  $\mu$ g tRNA, 30  $\mu$ g of nuclear or cytoplasmic extract and 100,000 cpm of p21WAF1 3' UTR RNA probe and incubated at room temperature for 1 hour. RNase T1 was added to the binding reactions and incubated at 37°C for 15 minutes. A 6% acrylamide gel containing 2.5% glycerol was prepared in Tris-Glycine Buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) for the RMSA. Binding complexes were resolved on the native gel at 120 volts for approximately 3 hours in a PROTEAN II xi Cell. The gel was dried in a Gel Dryer Model 583 and visualized by the Molecular Imager FX.

## **4. RESULTS**

### **4.1. THE EFFECT OF HISTONE DEACETYLASE INHIBITORS ON THE GENE EXPRESSION LEVELS OF C-SRC**

#### **4.1.1. The Influence of Histone Deacetylase Inhibitors on Tumor Cell Growth**

HDIs have been shown to harbor potent anti-tumor activities in a range of human tumor cells and animal models, and as such, have promising therapeutic value (Marks *et al.*, 2001, Vigushin and Coombes, 2002 and Arts *et al.*, 2003). One HDI that inhibits cellular growth of tumor cells is sodium butyrate (NaB), a non-competitive HDI that exists at physiologically relevant concentrations within the colon as a byproduct of anaerobic bacterial fermentation of dietary fiber (Sealy and Chalkley, 1978, Roediger, 1980 and Carey and La Thangue, 2006).

Previous work performed in the Bonham Lab has identified differential SRC promoter usage and relative c-Src expression levels across a variety of tumor cell lines (Bonham *et al.*, 2000, Dehm *et al.*, 2001 and Dehm and Bonham, 2004b). To investigate the effect of HDIs on c-Src expression levels, four cell lines were chosen based on the criteria of cellular origin, SRC promoter usage and c-Src expression levels. Two colon cancer cell lines were selected (HT29 and SW480), as well as one breast cancer cell line (T-47D) and one hepatocellular carcinoma cell line (HepG2). HT29 cells were chosen based on their utilization of both the SRC1A and SRC1 $\alpha$  promoters, and relatively high c-Src mRNA and protein expression levels. In contrast, SW480 cells primarily synthesize mRNA transcripts from the SRC1A promoter and exhibit low c-Src expression levels. T-47D cells also employ the SRC1A promoter but express c-Src at moderate levels, while HepG2 cells predominantly use the SRC1 $\alpha$  promoter and display high c-Src expression levels.

To monitor the anti-proliferative effects of NaB, the growth kinetics of HT29 and SW480 colon cancer cells were studied over 24, 48, 72 and 96 h time periods following exposure to NaB doses ranging from 0 - 10.0 mM (Figure 4.1.A, B). Growth curve analysis indicated that NaB represses colon tumor cell proliferation of HT29 and SW480 cells in a dose dependent manner and that a dose of 5.0 mM NaB was sufficient to completely inhibit colonic cell growth. Similarly, NaB affected cell growth of T-47D cells (Figure 4.1.C), however, a dose as low as 1.0 mM NaB caused a significant decrease in cell proliferation and 2.0 mM NaB prevented cell growth entirely.

Given that NaB is quite dissimilar from other HDIs, including Trichostatin A (TSA), the anti-proliferative effects of NaB and TSA were studied side by side in HepG2 hepatocellular carcinoma cells (Figure 4.2.A, B). HepG2 cells were cultured alone or following treatment with NaB concentrations ranging from 0.5 mM – 10.0 mM or TSA from 0.5  $\mu$ M – 10.0  $\mu$ M for 24, 48, 72, and 96 h. Once again, cellular growth of HepG2 cells decreased in a dose dependent manner following exposure to both NaB and TSA. Similar to T-47D cells, a dose of 1.0 mM NaB was capable of inhibiting cellular proliferation and 2.0 mM NaB was optimal for stopping the growth of HepG2 cells. TSA, on the other hand, inhibited cell growth at significantly lower concentrations, where 1.0  $\mu$ M TSA was sufficient for the absence of HepG2 cellular proliferation. These results conform with the current literature, that TSA is a much more potent HDI than NaB and is capable of inducing cellular growth arrest at  $\mu$ M concentrations, instead of the mM concentrations that NaB requires (Rosato and Grant, 2004). Furthermore, 5.0 mM NaB and 1.0  $\mu$ M TSA appear to be the optimal doses required to convincingly abrogate tumor cell growth. The viability of HepG2 cells at the optimal NaB and TSA doses were confirmed following 96 hours of HDI treatment by trypan blue staining (results not shown).

#### **4.1.2. Histone Deacetylase Inhibitor Downregulation of c-Src Expression Levels**

Upon selecting a diverse panel of tumor cell lines, and establishing the concentration range required for the anti-proliferative effects of NaB and TSA in these cell lines, HDI dose response experiments were performed. Principally, the action of NaB was monitored in HT29 and SW480 human colon cancer cells (HCCLs), given that

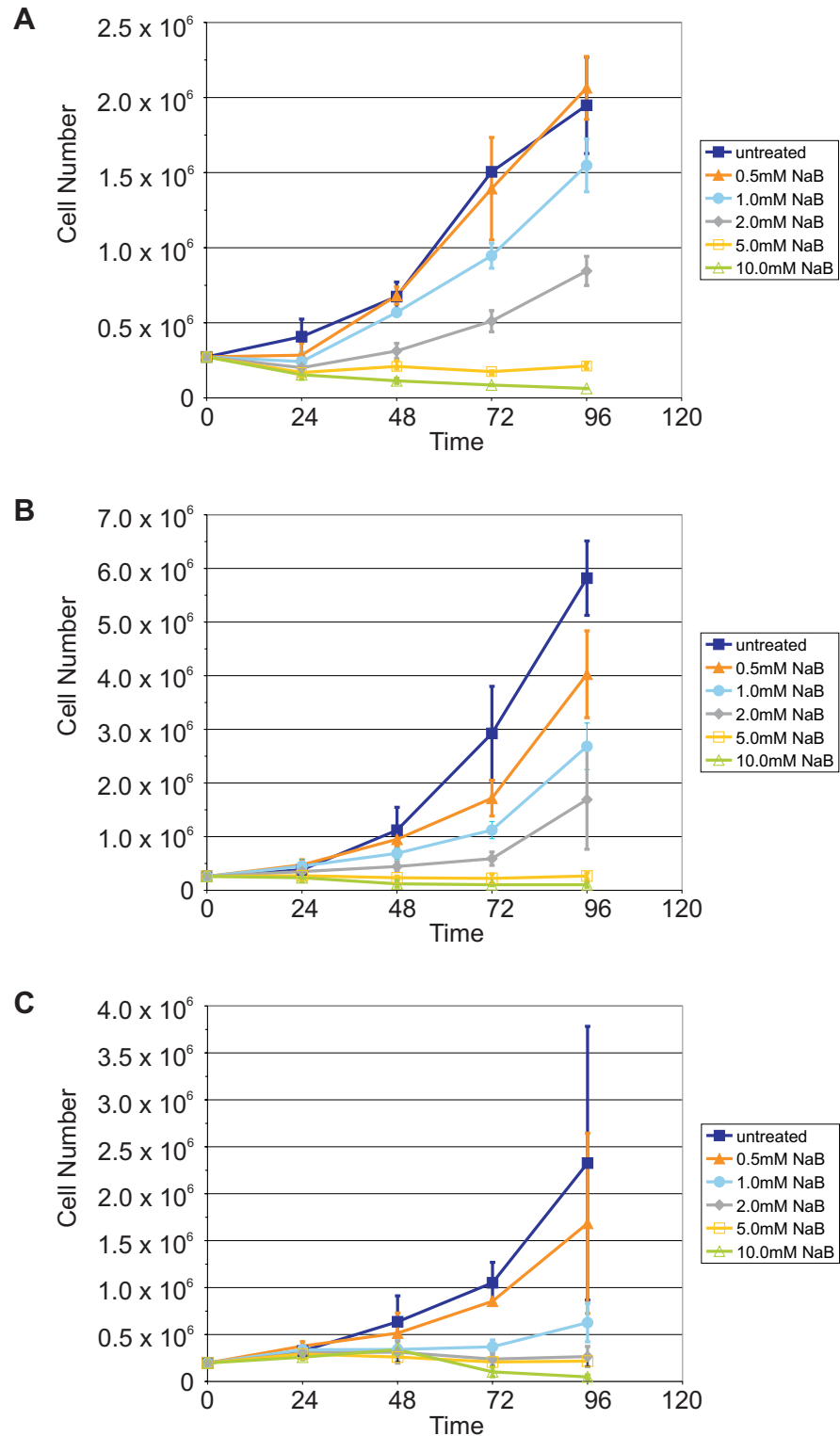


Figure 4.1. Sodium butyrate decreases cell growth in human colon and breast tumor cells. Growth curve analysis of (A) HT29, (B) SW480, and (C) T-47D cells following 96 h of treatment with increasing concentrations of NaB ranging from 0 - 10.0 mM. The data represent an average of two independent experiments performed in duplicate and include error bars for standard deviations.

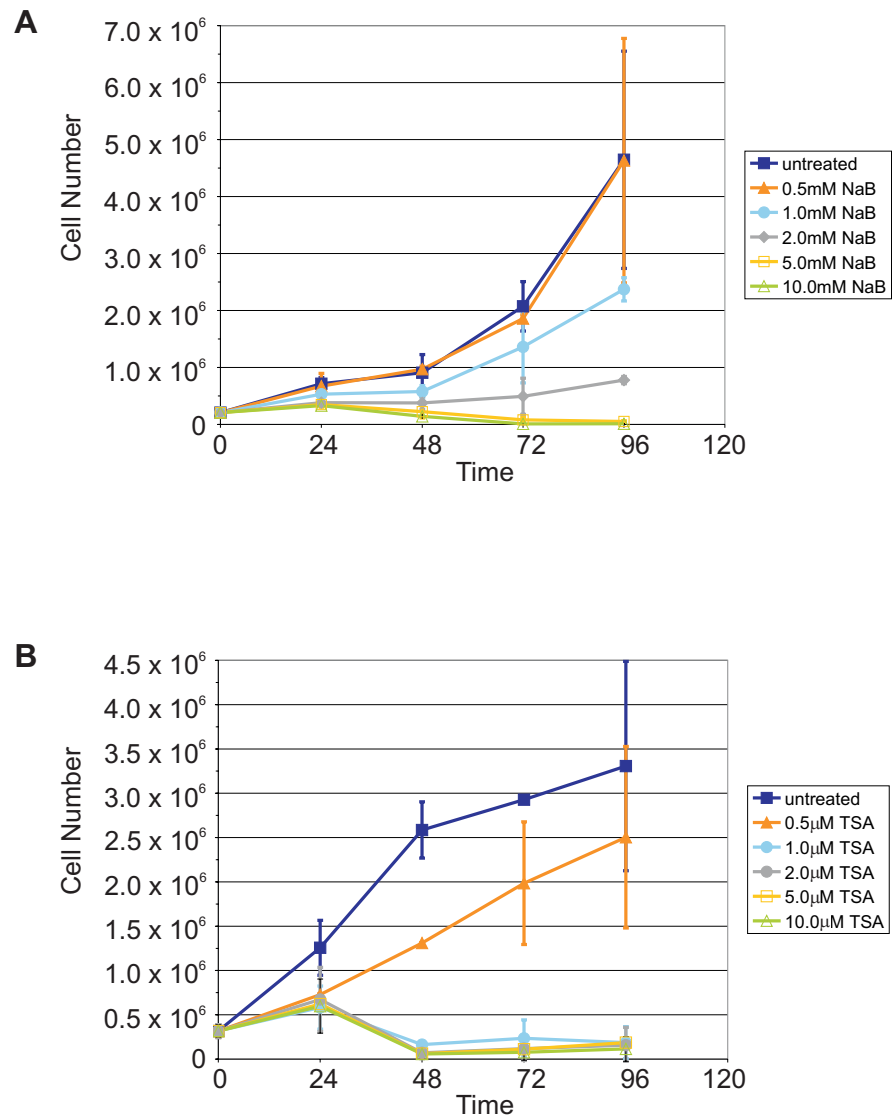


Figure 4.2. Histone deacetylase inhibitors decrease cell growth in HepG2 cells. Growth curve analysis of HepG2 cells treated with increasing doses of either (A) 0 - 10.0 mM NaB or (B) 0 - 10.0  $\mu$ M TSA for a 96 h time period. The data represent an average of two independent experiments, each performed in duplicate and include error bars for standard deviations.

physiologically high butyrate concentrations are naturally produced in the colon and c-Src protein levels and/or kinase activity is frequently elevated in malignant colonocytes compared to normal colonic mucosa (Roediger, 1980, Rosen *et al.*, 1986, Bolen *et al.*, 1987, and Cartwright *et al.*, 1989). Remarkably, NaB dose response experiments revealed that c-Src mRNA expression levels were repressed in a dose dependent manner by an effective dose of 2.0 – 10.0 mM NaB for 24 h in HT29, and SW480 cells (Figure 4.3.A, B). c-Src mRNA levels of T-47D and HepG2 cells were also consistently downregulated within this optimal dose range (Figure 4.3.C, D). Since p21WAF1 is a well defined target of HDIs, all dose response Northern blots were re-probed with p21WAF1 as a positive control (Nakano *et al.*, 1997, Sowa *et al.*, 1997 and Huang *et al.*, 2000). Accordingly, 2.0 – 10.0 mM NaB was observed to induce p21WAF1 mRNA expression levels in HT29, SW80, T-47D and HepG2 cells (Figure 4.3.). Taking these observations into account, an average dosage of 5.0 mM was chosen to further study NaB mediated c-Src downregulation.

Time course experiments carried out with the HT29, SW480, T-47D and HepG2 cell lines revealed that 5.0 mM NaB was capable of significantly repressing c-Src and elevating p21WAF1 mRNA levels as early as 6 h following treatment (Figure 4.4.A). In addition, NaB was also shown to suppress c-Src mRNA expression levels completely in the metastatic breast carcinoma cell lines MDA-MB-231 and MCF7 after 24 h of exposure (Figure 4.4.A). It was noted from both the dose response and time course experiments that NaB appeared to inversely regulate c-Src and p21WAF1, and often the RNA sample with the lowest level of c-Src also displayed the highest level of p21WAF1 mRNA expression.

Subsequently, to establish if decreases in c-Src mRNA were matched by a drop in c-Src protein levels, Western blot analysis was performed with HT29, SW480, T-47D and HepG2 cell NaB time course samples. Similar to c-Src mRNA, NaB downregulated c-Src protein levels in a time dependent fashion. However, suppression of c-Src protein expression levels was expectantly delayed as decreases in protein expression were not apparent until the cells had been exposed to NaB for a minimum of 18 h (Figure 4.4.B). To ensure that the NaB mediated downregulation of c-Src levels was not a consequence of pleiotropic effects beyond inhibiting HDACs, the effect of



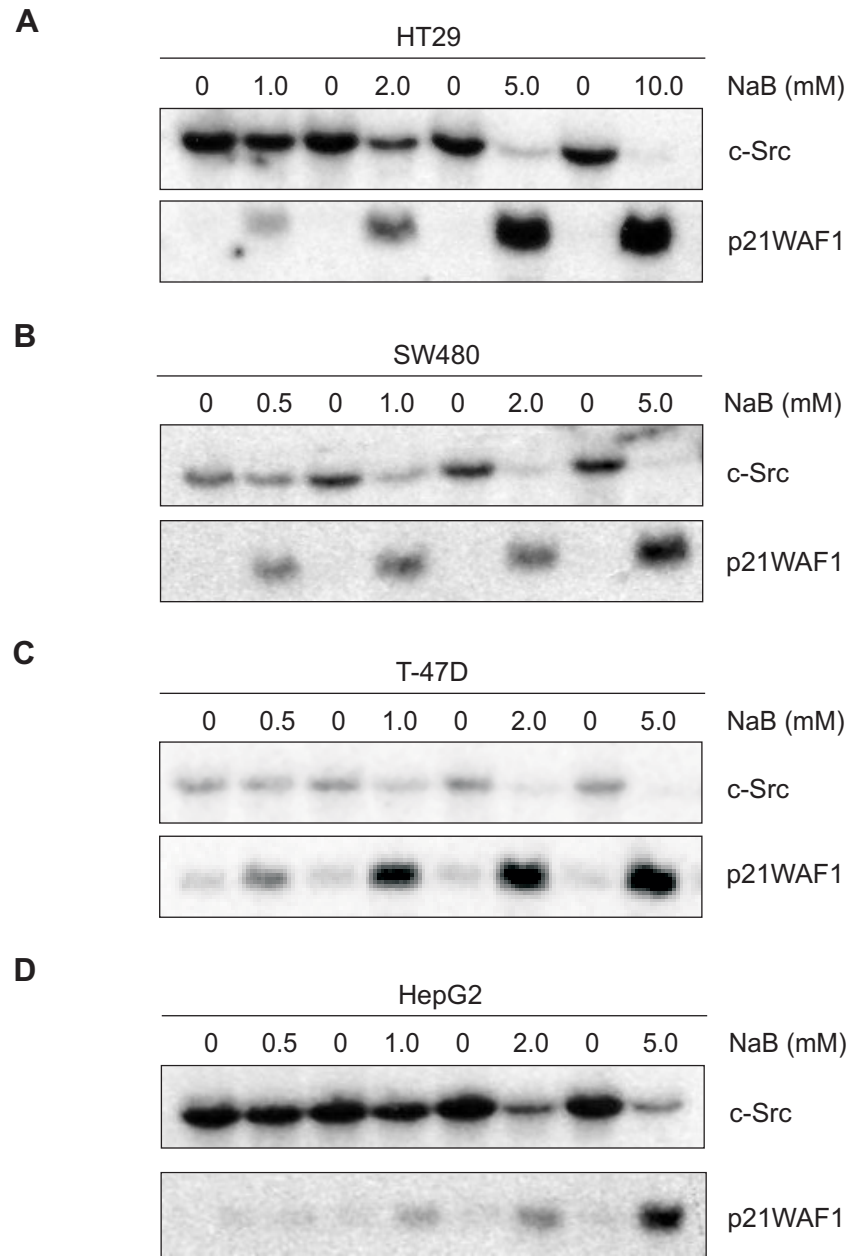


Figure 4.3. Sodium butyrate suppresses c-Src mRNA levels dose dependently in human tumor cells. Northern blot analysis was performed on total RNA extracted from (A) HT29, (B) SW480, (C) T-47D, and (D) HepG2 cells following treatment with NaB doses ranging from 0 - 10.0 mM for a 24 hour time period. The mRNA levels of c-Src and p21WAF1 were examined. The data shown are a representation of three experiments.

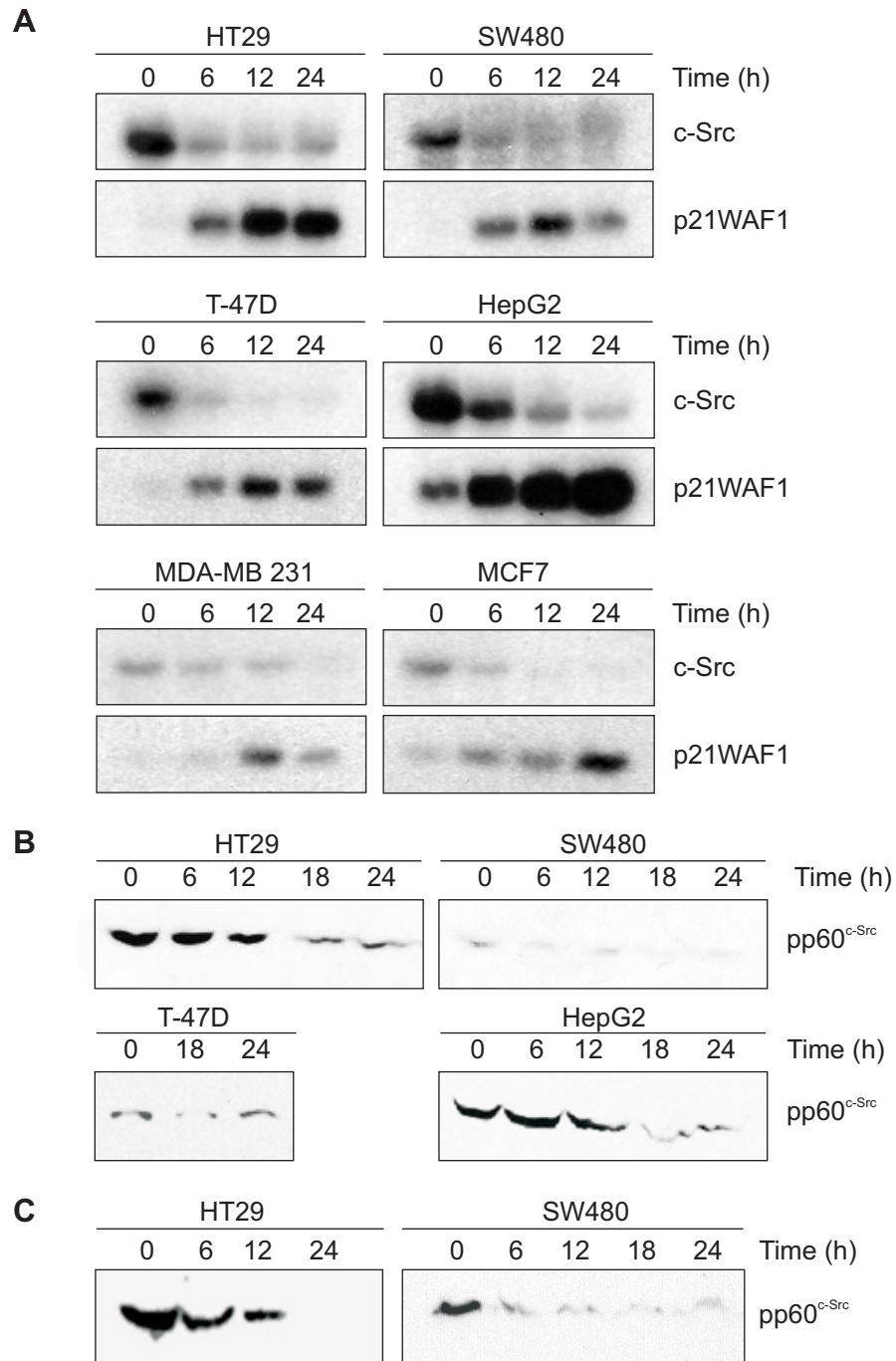


Figure 4.4. c-Src mRNA and protein levels are repressed by histone deacetylase inhibitors in human tumor cells. A. Total RNA was extracted from HT29, SW480, T-47D, HepG2, MDA-MB-231 and MCF-7 cells following treatment with 5.0 mM NaB for 0 - 24 h and Northern blot analysis was performed with c-Src and p21WAF1 probes. B. Total cellular protein was harvested from HT29, SW480, T-47D and HepG2 cells after 0 - 24 h of NaB treatment and analyzed by Western blot for c-Src protein (pp60<sup>c-Src</sup>). C. Western blots were performed on HT29 and SW480 cells treated with 1.0  $\mu$ M TSA for 0 - 24 h and analyzed for pp60<sup>c-Src</sup> levels. The Northern blot data shown are a representation from three independent experiments, while the Western blot data are a representative from two experiments.

TSA, a competitive HDI, on c-Src protein levels was monitored. Clearly, 1.0  $\mu$ M TSA also inhibited the expression of c-Src protein levels in HT29 and SW480 cells, over 24 h (Figure 4.4.C). Taken together, these observations suggest that HDIs may universally downregulate c-Src expression, regardless of tissue, SRC promoter usage or relative c-Src mRNA and protein levels.

#### **4.1.3. The Effect of Histone Deacetylase Inhibitors on SRC Transcription**

In view of the observation that c-Src expression levels are inhibited by NaB in cell lines which display a preference for either the SRC1 $\alpha$  promoter (HepG2) or SRC1A promoter (SW480), the potential of HDIs to suppress activity of both SRC promoters was assessed. For these experiments, the minimal SRC1 $\alpha$  (-145SRC1 $\alpha$ -CAT) promoter plasmid, harboring the HNF-1 site, was transiently transfected into HepG2 cells and treated with NaB (5.0 mM) or left untreated for 24 h (Figure 4.5.A). Similarly, the minimal SRC1A (0.38SRC1A-CAT) promoter vector, containing the *cis*-acting GC1, GA2, TC1, TC2 and TC3 sites, was transiently transfected into HepG2 cells and exposed to NaB in the same manner (Figure 4.5.B). CAT levels were used to determine the activity of the -145SRC1 $\alpha$  and 0.38SRC1A promoters in the absence and presence of NaB treatment. Interestingly, NaB repressed both the highly dissimilar SRC1 $\alpha$  and SRC1A promoters by approximately 80%, in HepG2 cells. Furthermore, 1.0  $\mu$ M TSA inhibited the SRC1 $\alpha$  and SRC1A promoters by 90% in HepG2 cells (Kostyniuk *et al.*, 2002). Identical -145SRC1 $\alpha$ -CAT and 0.38SRC1A-CAT plasmid transient transfections carried out in SW480 cells with NaB and TSA also displayed a respective 90% and 95% decrease in the activity of both promoters (Kostyniuk *et al.*, 2002). To ensure that the transient transfections were working correctly, the promoter activity of p21WAF1 was assessed. Three p21WAF1 promoter vectors were tested: the full length -2300WAF1-CAT vector, which possessed all six Sp binding sites and two distal p53 REs; the -210WAF1-CAT reporter plasmid, which lacked the p53 REs but still contained the Sp elements; and the -101WAF1-CAT vector, which harbored only Sp sites 3-6. Transient transfections with these reporter plasmids in HT29 cells revealed that -210WAF1-CAT displayed the highest basal promoter activity of the WAF1-CAT promoter vectors, and that both NaB and TSA were competent for promoter

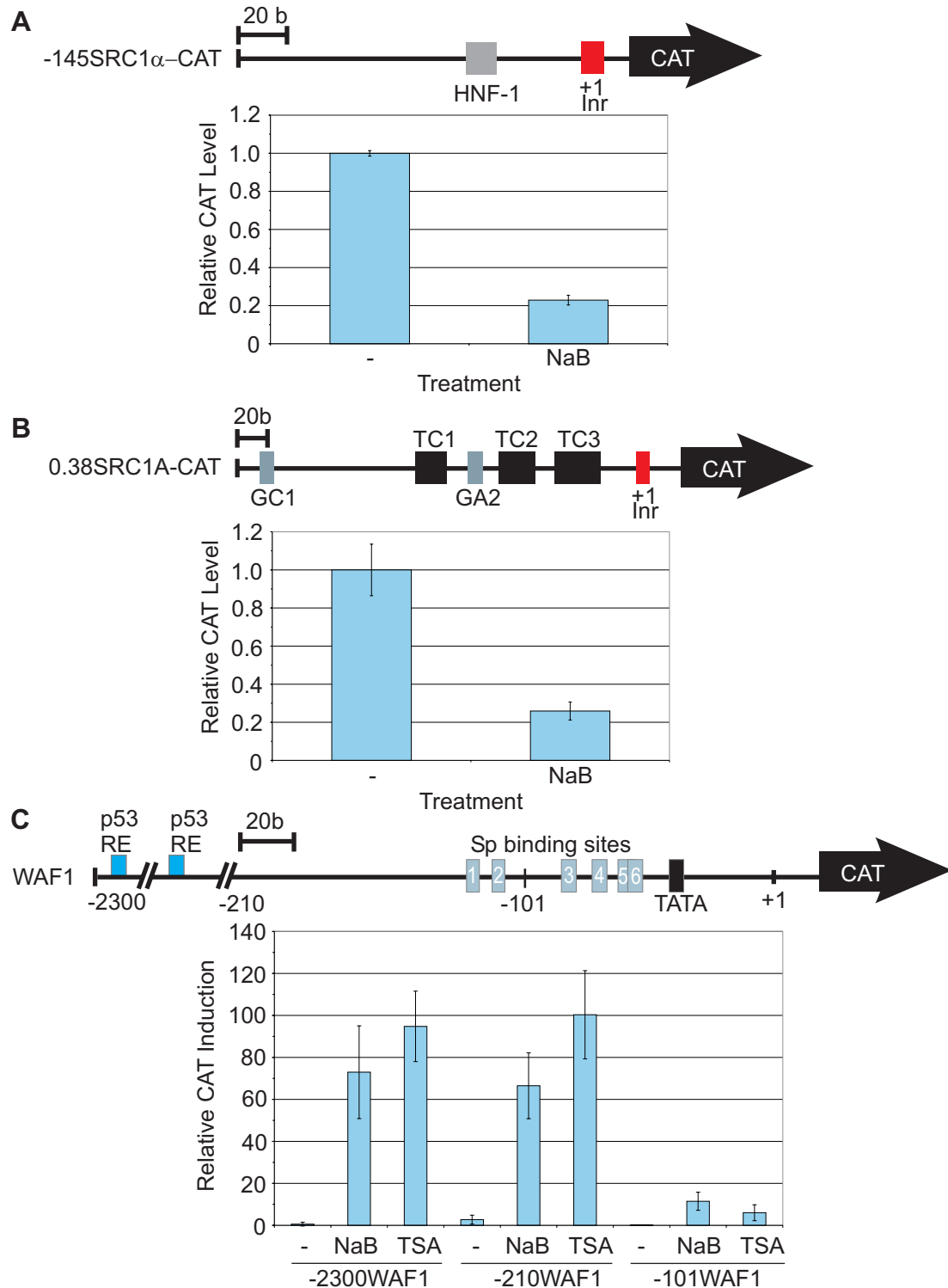


Figure 4.5. Sodium butyrate mediated repression of the SRC1 $\alpha$  and SRC1A promoters. HepG2 cells were transfected with (A) 0.38SRC1A-CAT or (B) -145SRC1 $\alpha$  promoter constructs and treated with or without NaB (5.0 mM) for 24 h prior to assessing CAT levels. C. The -2300, -210, or -101WAF1-CAT promoter constructs were transfected into HT29 cells and treated with either NaB (5.0 mM) or TSA (1.0  $\mu$ M) for 24 h. Each of these experiments was performed in duplicate a minimum of two times. The error bars are a representation of standard deviations.

upregulation of all three WAF1-CAT reporter plasmids (Figure 4.5.C). Therefore, HDIs appear to inhibit c-Src expression levels by suppressing the SRC1 $\alpha$  and SRC1A promoters and to elevate p21WAF1 expression levels by activating the WAF1 promoter.

#### **4.1.4. p21WAF1 Activation of SRC Transcription**

Given that c-Src downregulation inversely correlates with p21WAF1 upregulation and HDIs have been shown to suppress activity of the SRC promoters, a role for p21WAF1 in HDI mediated repression of the SRC1 $\alpha$  and SRC1A promoters was explored. Transient co-transfections were performed in SW480 and HepG2 cells with the reporter plasmid -145SRC1 $\alpha$ -CAT and a p21WAF1 expression vector pCMV-Cip1 to determine if p21WAF1 had the ability to suppress the SRC1 $\alpha$  promoter. Contrary to this hypothesis, pCMV-Cip1 trans-activated the SRC1 $\alpha$  promoter nearly 2-fold in SW480 cells and 4-fold in HepG2 cells (Figure 4.6.A). When HDIs were added to the co-transfections, -145SRC1 $\alpha$  promoter levels were subsequently repressed. Furthermore, the 0.38SRC1A promoter was stimulated by pCMV-Cip1 in HepG2 cells, but not in SW480 cells, and in every instance HDIs were still proficient at repressing the SRC1A promoter (Figure 4.6.B). Hence, p21WAF1 is not responsible for HDI facilitated downregulation of the SRC1 $\alpha$  and SRC1A promoters. However, p21WAF1 does appear to activate these promoters by a novel mechanism, which may be overridden by HDIs.

#### **4.1.5. Downregulation of c-Src Expression Levels by HDIs does not Require New Protein Synthesis**

To investigate whether HDIs directly or indirectly inhibit c-Src expression levels, additional time course experiments were carried out with the protein synthesis inhibitor cycloheximide (CHX). Four selected cell lines were subjected to time course analysis with NaB (5.0 mM), CHX (50  $\mu$ M), or NaB (5.0 mM) and CHX (50  $\mu$ M) jointly. In HT29, SW480, T-47D, and HepG2 cells, NaB treatment alone repressed c-Src mRNA levels, as previously reported, and CHX treatment induced c-Src mRNA expression (Figure 4.7.A-D). However, when the cells were exposed to both agents,

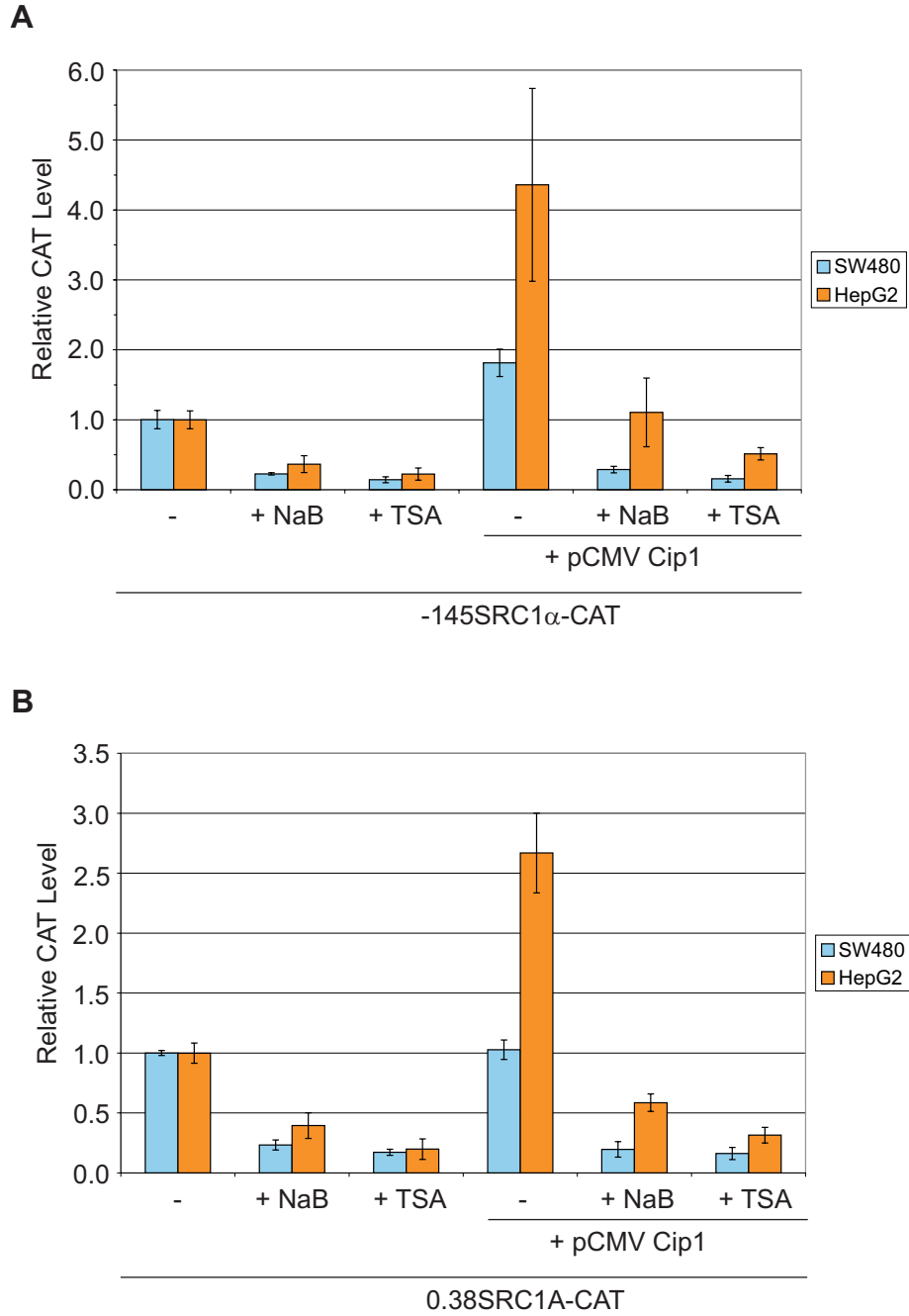


Figure 4.6. The SRC1 $\alpha$  and SRC1A promoters are trans-activated by p21WAF1 in SW480 and HepG2 cells. Cells were transiently transfected with (A) 0.38SRC1A-CAT or (B) -145SRC1 $\alpha$  promoter constructs in the absence or presence of co-transfected pCMV Cip1 and left untreated (-) or exposed to NaB (5.0 mM) or TSA (1.0  $\mu$ M) for 24 h. These data are the average of two independent experiments, each carried out in duplicate and include error bars for standard deviations.

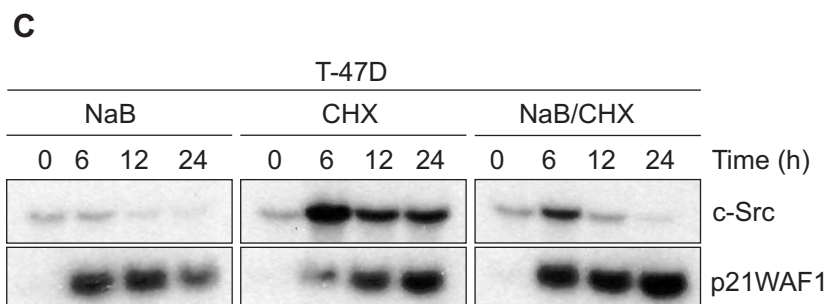
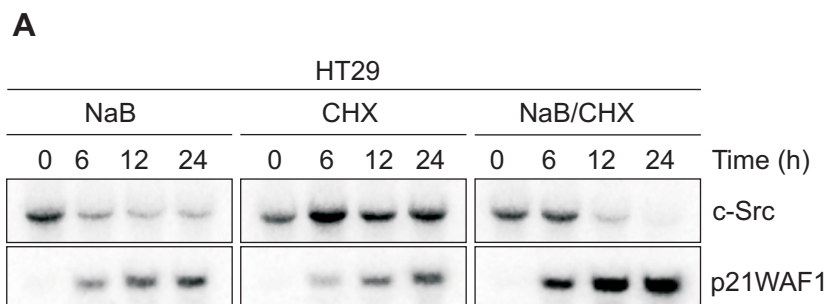


Figure 4.7. c-Src mRNA levels are reduced by sodium butyrate independent of new protein synthesis. Total RNA was extracted from HT29 (A), SW480 (B), T-47D (C) and HepG2 (D) cells. Cells were treated for the allotted time frames with NaB (5.0 mM), CHX (50  $\mu$ M), or both NaB (5.0 mM) and CHX (50  $\mu$ M) together. RNA levels of c-Src and p21WAF1 were examined by Northern blot. Each experiment was performed a minimum of two times.

CHX was unable to prevent HDI mediated repression of c-Src in all cell lines examined. Moreover, when Northern blots were re-probed with p21WAF1, NaB and CHX alone elevated p21WAF1 mRNA levels, while the combination of NaB and CHX stimulated p21WAF1 expression even further in HT29, SW480 and T-47D cells (Figure 4.7.A-C). In HepG2 cells, therefore, p21WAF1 mRNA expression was not upregulated by NaB in the presence of CHX (Figure 4.7.D). These results suggest that HDIs suppress c-Src expression independent of new protein synthesis. Furthermore, the observation that protein neo-synthesis is not necessary for HDIs to elevate p21WAF1 mRNA levels in HT29, SW480 and T-47D cells conforms with the current literature (Nakano *et al.*, 1997 and Sowa *et al.*, 1997). The need for new protein synthesis to enhance the mRNA expression levels of p21WAF1 in HepG2 cells, therefore, is a novel finding that will be discussed in greater detail in the third part of the results section.

#### **4.1.6. SUMMARY**

c-Src overexpression has been implicated in tumor development, growth and metastasis. Therefore, the potential of HDIs to affect c-Src gene expression in colon carcinoma cells was investigated. HDIs were capable of efficiently inhibiting c-Src mRNA and protein levels in a dose and/or time dependent manner, not only in HCCLs, but also in breast and hepatocellular carcinoma cell lines. Surprisingly, this repression of c-Src levels was suggested to be the result of HDI mediated downregulation of the SRC promoters, as both the dissimilar SRC1 $\alpha$  and SRC1A promoters were repressed by HDIs to a similar degree. Furthermore, HDIs were observed to regulate c-Src expression independent of new protein synthesis. Therefore, these results suggest that c-Src may be an important target of HDIs, and in particular of butyrate, which physiologically resides in the colon at equivalent dosages to those utilized in the above experiments (Kostyniuk *et al.*, 2002).



## **4.2. THE RESPONSE OF C-SRC FAMILY KINASE MEMBERS TO HISTONE DEACETYLASE INHIBITORS**

### **4.2.1. The Relative Expression Patterns of c-Src Family Kinase Members in HCCLs.**

In addition to overexpression and/or activation of c-Src, elevated gene expression level and kinase activity of a number of the c-Src family kinase (SFK) members have been reported in tumor development, proliferation, motility, invasiveness and progression (Biscardi *et al.*, 1999; Frame, 2002; Russello and Shore, 2003 and Summy and Gallick, 2003). Furthermore, experiments performed with SFK knockout mice have suggested SFKs may elicit compensatory effects for one another, likely due to their high amino acid sequence conservation (Lowell and Soriano, 1996 and Stein *et al.*, 1994). Therefore, given the potential for functional redundancy among the members, and that acquired deregulation of a subset of SFK members appears to be implicated in colorectal carcinoma development and progression, the ability of NaB to inhibit the expression of other SFK members expressed in HCCLs was tested.

Although a complete survey of the relative c-Src mRNA and protein expression levels in various HCCLs has been reported, a comprehensive analysis of the comparative expression patterns of the remaining SFK members had not been studied (Dehm *et al.*, 2001). Nine representative HCCLs (Colo 201, Colo 205, Colo 320, SW480, SW620, HT29, DLD-1, WiDr and LS174T) were chosen to carry out this objective and establish the gene expression levels of the SFKs. mRNA levels of five SFKs, c-Src, Yes, Fyn, Lck and Lyn were expressed in at least four of the HCCLs examined, while expression of Hck, Fgr and Blk was not detected in any of the HCCLs (Figure 4.8. and data not shown). Interestingly, the cell lines displayed quite distinct SFK expression signatures, which could be grouped together based on similarities in mRNA expression levels. For example, the related cell lines Colo 201 and Colo 205 exhibited relatively high levels of c-Src, Lck and Lyn gene expression, but had significantly lower levels of Yes and Fyn. Conversely, the isogenic SW480 and SW620 cell lines expressed Lck, Fyn, and Lyn at high levels, but not c-Src or Yes. Furthermore, HT29, WiDr and LS174T cells displayed a similar pattern of high c-Src and Yes mRNA expression, although LS174T also expressed higher levels of Lyn. Colo 320 cells, on

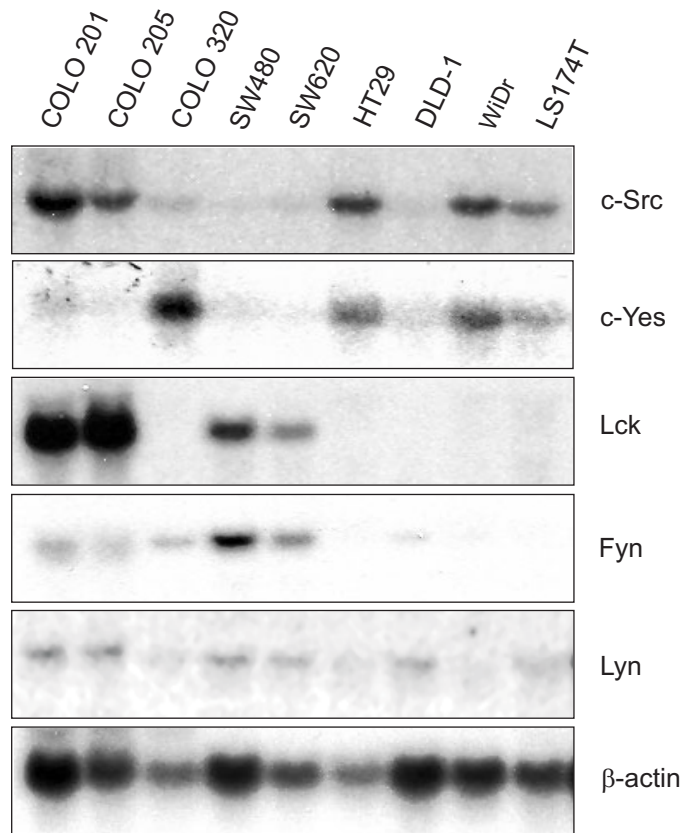


Figure 4.8. Differential gene expression of c-Src family kinase members in human colon cancer cell lines. Total RNA was harvested from nine colon tumor cell lines and Northern blot analysis of total RNA was probed for the SFK members, c-Src, Yes, Lck, Fyn and Lyn.

the other hand, had a uniquely high level of Yes mRNA expression, but did not express any other SFKs to the same degree. Generally speaking, the relative level of specific SFK mRNA expression was mirrored by its protein levels (Hirsch *et al.*, 2006). Based on these similarities in the pattern of SFK member gene expression, four representative cell lines were chosen for further study: Colo 201, Colo 320, SW480 and HT29.

#### **4.2.2. Histone Deacetylase Inhibitors Decrease c-Src Family Kinase Expression Levels**

To begin examining HDI mediated effects on the SFK members, NaB dose response experiments were performed in HT29, Colo 320, SW480 and Colo 201 cells (Figure 4.9.A-D). The effect of NaB on the mRNA expression level of each SFK member was examined in at least two different HCCLs. For example, since c-Src is overexpressed in HT29 and Colo 201 cells, dose response experiments to examine HDI mediated effects on c-Src mRNA expression levels were carried out in both of these cell lines. As previously observed the presence of NaB suppressed c-Src mRNA levels in a dose dependent manner in HT29 and Colo201 cells. A repression of other SFKs was also observed upon examination. Lck mRNA expression levels were repressed in a dose dependent manner in both Colo 201 and SW480 cells. Similarly, Fyn mRNA levels were dose dependently repressed by NaB in SW480 cells and to a lesser extent in Colo 320 cells, which express relatively low endogenous levels of Fyn to begin with (Figure 4.8). Yes mRNA expression levels were also downregulated in HT29 cells, and less strikingly in Colo 320 cells, over the 24 h treatment period. However, a curious reproducible transient induction was noted in Yes mRNA levels, which was especially evident in the Colo 320 cells. Lyn mRNA levels were also transiently induced, particularly in Colo 201 cells, followed by an NaB mediated dose dependent repression over the remaining time course. In comparison, the NaB mediated downregulation of Lyn expression observed in SW480 cells was less dramatic. However, in contrast to the general repression of SFK members, the expression level of p21WAF1 was induced in all cell lines (Figure 4.9.B, C) (Kostyniuk *et al.*, 2002). Therefore, these observations suggested that NaB may influence the mRNA expression of SFK members in somewhat of a cell specific manner, although NaB appears to be capable of inhibiting mRNA

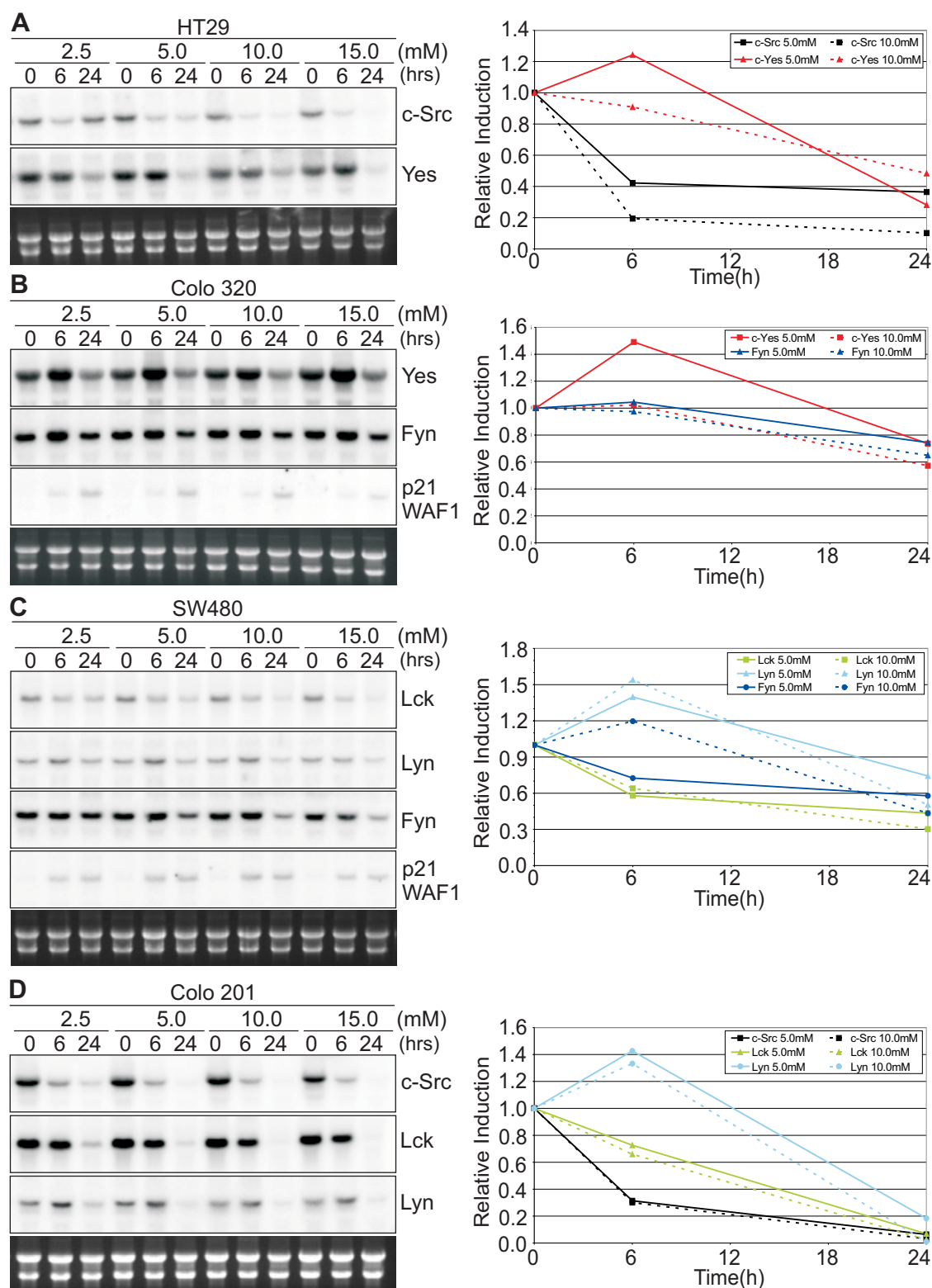


Figure 4.9. Sodium butyrate inhibits mRNA expression of the c-Src family kinase members in human colon cancer cell lines. Northern blots were performed with total RNA harvested from HCCLs (A) HT29, (B) Colo 320, (C) SW480 and (D) Colo 320 treated with either 2.5 mM, 5.0 mM, 10.0 mM, or 15.0 mM of NaB for 6 and 24 h or left untreated. Relative induction of SFKs was analyzed with a Molecular Imager and normalized to the RNA gel. A graphical representation of the 5.0 mM and 10.0 mM doses are shown for each HCCL.

expression levels of all SFK members expressed in HCCLs.

As NaB exhibits pleiotrophic effects in addition to being a HDI, time course experiments were also performed with TSA (1.0  $\mu$ M) in the four representative cell lines: SW480 (Figure 4.10.A), Colo 201 (Figure 4.10.B), HT29 (Figure 4.11.A), and Colo 320 (Figure 4.11.B) to evaluate changes in SFK mRNA expression. Once again, c-Src and p21WAF1 were used as positive controls. As expected, TSA downregulated c-Src and Lck mRNA levels in a time dependent manner in both SW480 and Colo 201 cells (Figure 4.11.A, B). Furthermore, Fyn mRNA levels were suppressed by TSA in SW480 and Colo 320 cells. Consistent with our earlier observations, Lyn mRNA levels were downregulated by TSA 24 h following exposure in SW480, Colo 201 and HT29 cells. However, the early transient induction in Lyn levels observed following NaB exposure was significantly less evident in HT29 cells treated with TSA. Yes mRNA expression levels still exhibited the same early transient induction followed by a sharp repression in mRNA levels after 24 h of incubation with TSA in Colo 320 colon tumor cells. However, TSA mediated a less convincing repression of Yes in HT29 cells, in contrast to NaB. Taken together, TSA appears to mimic the effects of the NaB dose response experiments over a 24 h treatment period, which further suggests that the HDI activity of NaB is responsible for the downregulation of SFK member mRNA expression.

#### **4.2.3. The Effect of Cycloheximide on Histone Deacetylase Inhibitor Mediated Repression of the c-Src Family Kinase Members**

Given that NaB was previously observed to repress c-Src mRNA levels independent of protein neo-synthesis, the requirement for new protein synthesis in HDI mediated downregulation of the remaining SFK members was examined (Kostyniuk *et al.*, 2002). Cycloheximide (50  $\mu$ M) time course experiments were carried out in the absence or presence of NaB (5.0 mM) in HT29 (A), Colo 320 (B), SW480 (C) and Colo 201 (D) cells (Figure 4.12.). Initially, the mRNA levels of Lck were assessed since the HDI response displayed by Lck was most similar to that observed by c-Src. In SW480 and Colo 201 cells, CHX alone induce a slight transient induction in Lck mRNA levels, while NaB was still capable of suppressing the mRNA expression levels of Lck in the

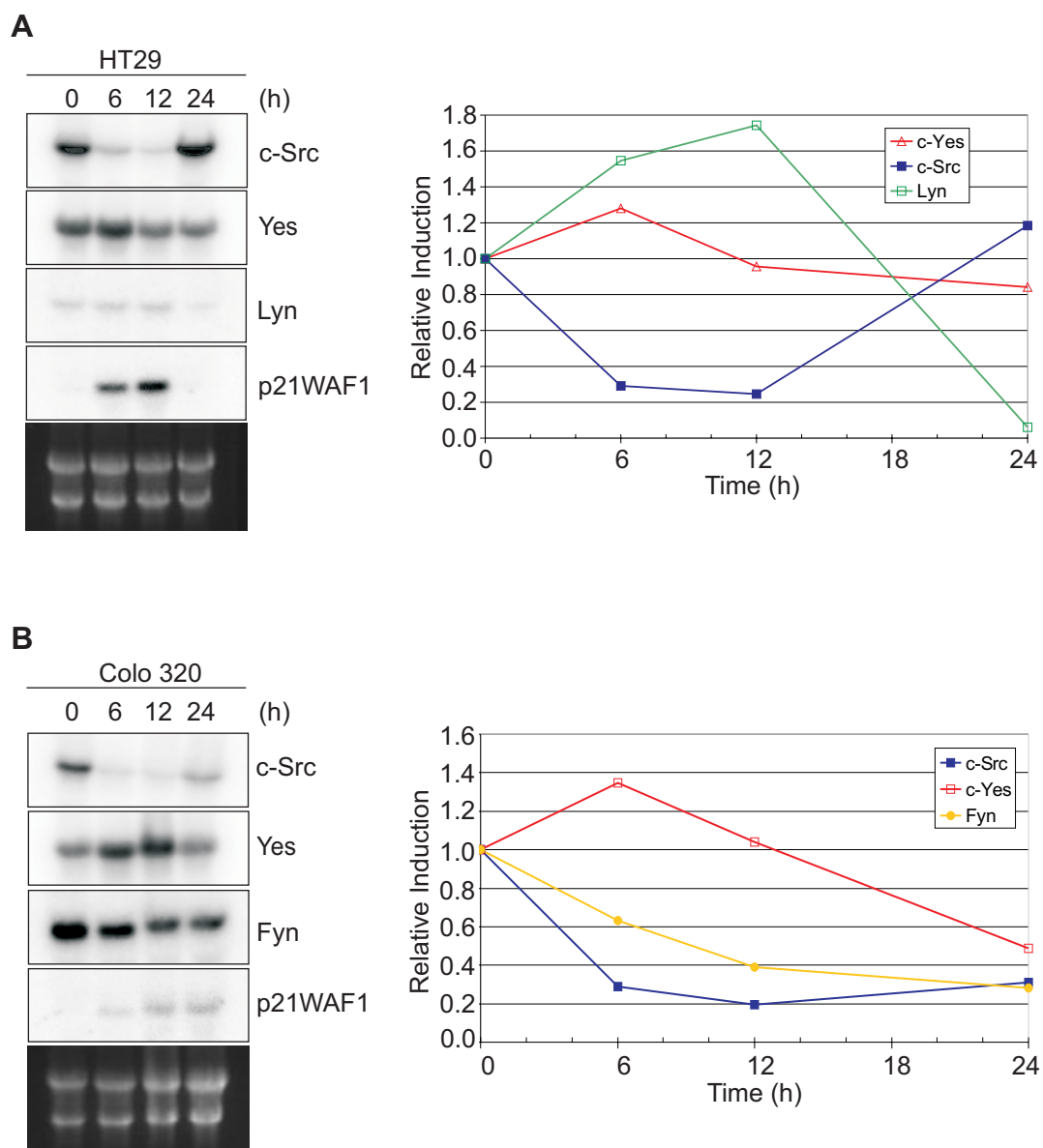


Figure 4.10. Trichostatin A represses the mRNA expression levels of the c-Src family kinase members in HT29 and Colo 320 cells. Total RNA was harvested from HCCLs treated with 1.0  $\mu$ M TSA for 6, 12 and 24 h or left untreated. Northern blots were performed on (A) HT29 and (B) Colo 320 cells and analyzed with a Molecular Imager to determine the mRNA expression levels of the SFK members and were normalized to the RNA gel to produce the graph. The Northern blots, along with the representative RNA gel for each HCCL are shown on the left.

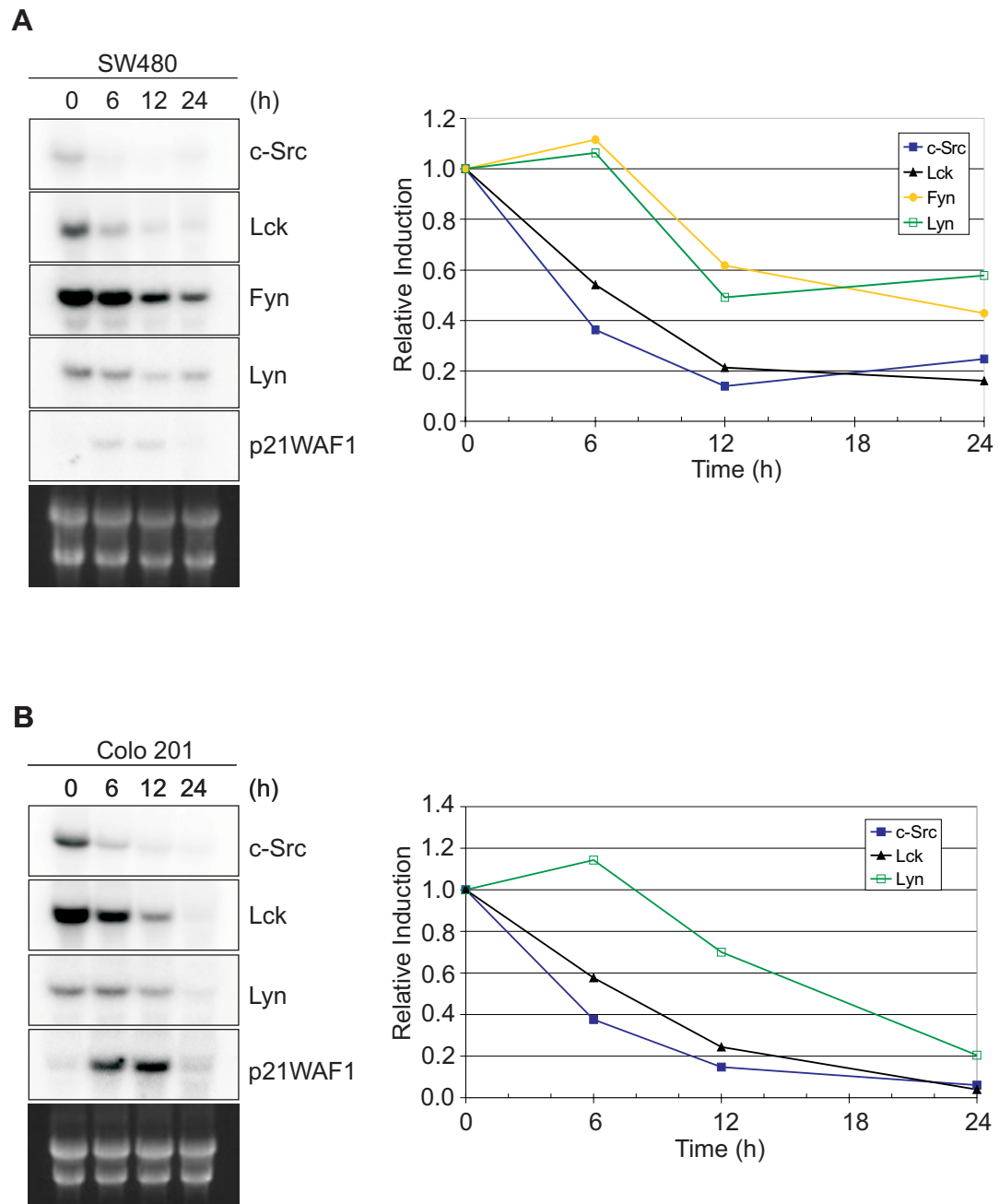


Figure 4.11. TSA inhibits the mRNA expression of SFK members in SW480 and Colo 201 cells. Total RNA was harvested from HCCLs treated with 1.0  $\mu$ M TSA for 6, 12 and 24 h or left untreated. Northern blots were performed on (A) SW480 and (B) Colo 201 cells and analyzed with a Molecular Imager to determine the mRNA expression levels of the SFK members and were normalized to the RNA gel to produce the graph. The Northern blots, along with the representative RNA gel for each HCCL are shown on the left.

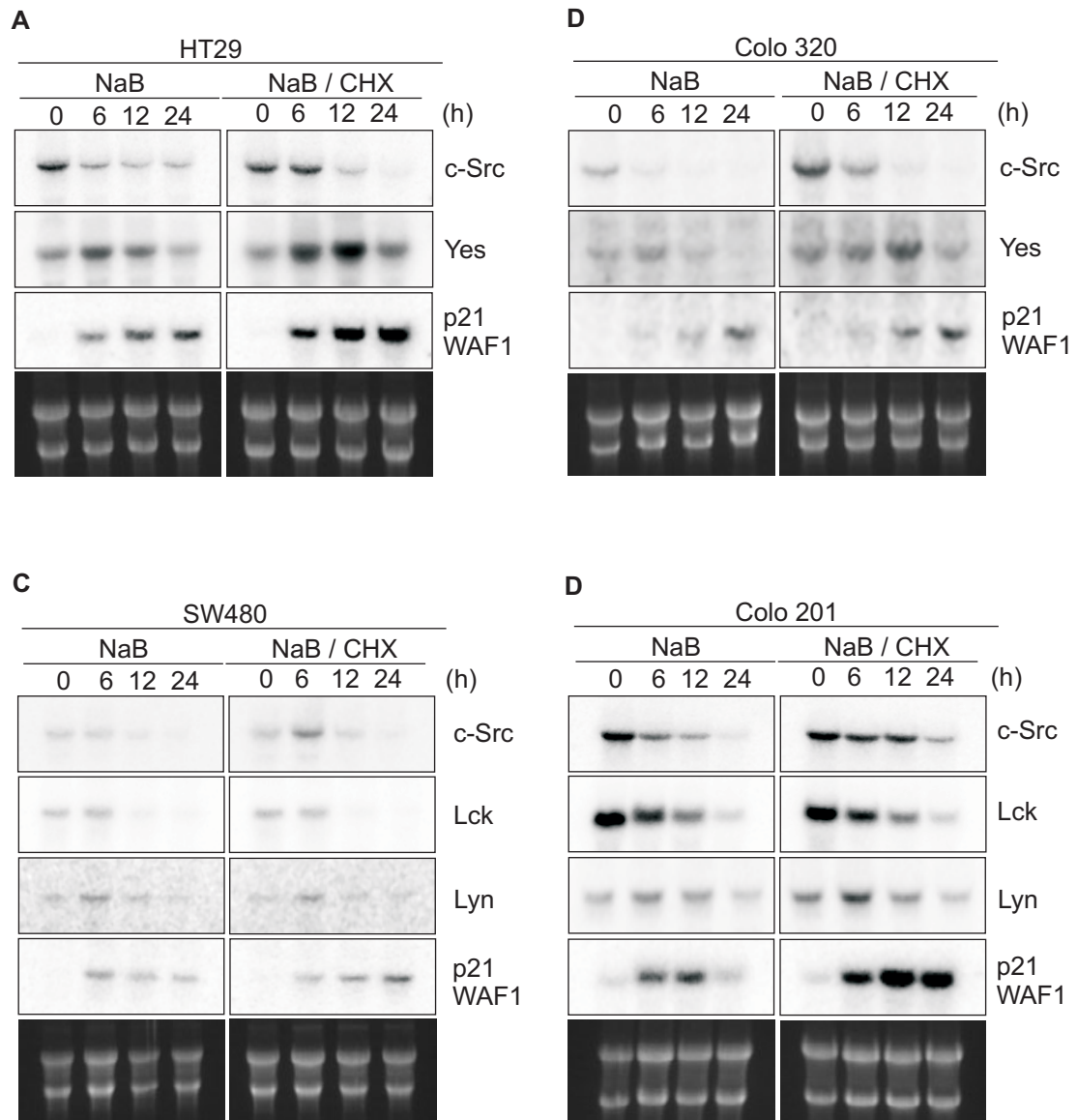


Figure 4.12. Sodium butyrate represses Lck and Lyn mRNA expression levels independent of protein neo-synthesis. Total RNA was collected from (A) HT29, (B) Colo 320, (C) SW480 and (D) Colo 201 cells treated with NaB (5.0 mM) in the presence or absence of a protein synthesis inhibitor, CHX (50  $\mu$ M) for 6, 12 and 24 h, and analyzed by Northern blot. HT29 and Colo 320 Northern blots were probed for c-Src, Yes and p21WAF1. SW480 and Colo 201 blots were probed for c-Src, Lck, Lyn and p21WAF1 levels.



presence of CHX, suggesting new protein is not a requirement (Figure 4.12 and data not shown). Similarly, protein neo-synthesis was not a prerequisite for HDI mediated repression of Lyn in SW480 or Colo 201 cells. The results for HDI mediated downregulation of Yes were inconclusive in Colo 320 and HT29 cells, given that CHX treatment alone resulted in a slight induction in Yes mRNA levels (Figure 4.12 and data not shown). As a control, all Northern blots were re-probed for p21WAF1, which exhibited a significant upregulation in mRNA expression upon joint exposure to NaB and CHX, as previously documented (Nakano *et al.*, 1997 and Sowa *et al.*, 1997). Therefore, in parallel to c-Src, HDIs directly downregulate Lck and Lyn levels in SW480 and Colo 201 cells, however, Yes mRNA levels may be indirectly regulated by HDIs.

#### **4.2.4. Promoters of c-Src Family Kinase Members are Downregulated by Histone Deacetylase Inhibitors**

Since NaB directly represses SFK mRNA expression levels of Lck and Lyn, transient transfections were performed to examine the transcriptional activity of the LCK type 1 and LYN promoters following treatment with HDIs. In addition, the effect of HDIs on the YES gene promoter was also monitored. The highly disparate SRC1A and SRC1 $\alpha$  promoters have been previously shown to be directly repressed by NaB and TSA, and therefore the SRC1A promoter was used as a positive control for these experiments (Kostyniuk *et al.*, 2002 and Dehm *et al.*, 2004). Transient transfections were carried out in SW480 cells with the 0.38SRC1A-CAT (A), -630LCK CAT (B), -590LYN-CAT (C) or 2.1YES-CAT (D) promoter constructs followed by treatment with either NaB (5.0 mM) or TSA (1.0  $\mu$ M) for 6, 12 or 24 hours (Figure 4.13.). The activities of the 0.38SRC1A, -630LCK and 2.1YES promoters were all repressed in a time dependent manner by as much as 80%. The -590LYN promoter was similarly suppressed in a time dependent fashion by TSA, however, while NaB downregulated the LYN promoter 6 h following treatment, the dramatic suppression that NaB imparted on the promoter was not maintained after 24 h of treatment. As a control, WAF1-CAT reporter plasmids were also upregulated in the presence of HDIs (data not shown). Taken together, these results suggest that HDIs are capable of directly and coordinately repressing SFK

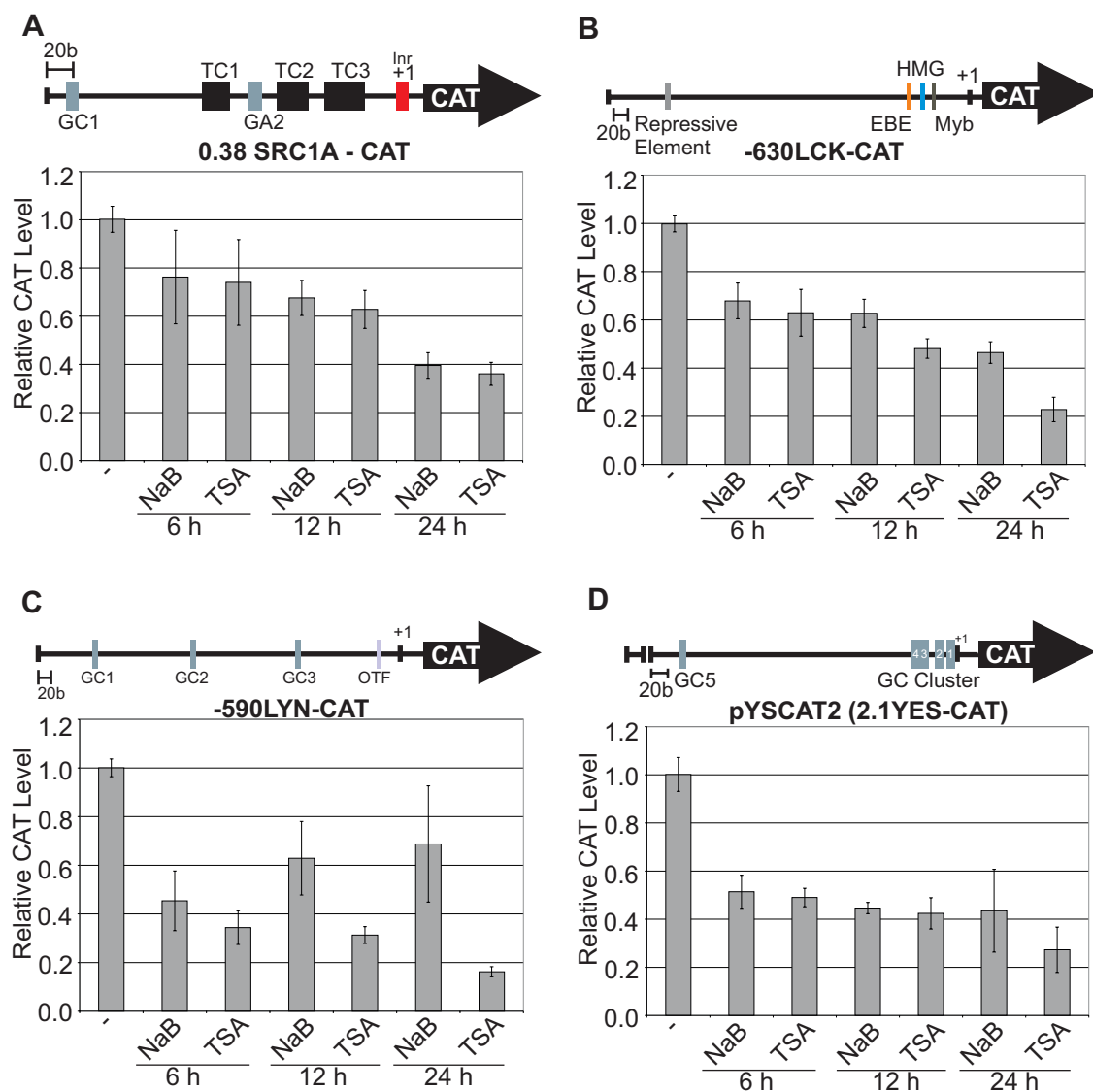


Figure 4.13. Histone deacetylase inhibitors downregulate the activity of the SRC1A, LCK type 1, LYN and YES promoters. SW480 cells were transfected with (A) 0.38SRC1A -CAT, (B) -630LCK-CAT, (C) -590LYN-CAT or (D) pYSCAT2 constructs in the presence of NaB (5.0 mM) or TSA (1.0  $\mu$ M). Transfected cells were harvested 6, 12 and 24 h following HDI treatment and CAT levels were measured. A cartoon of the respective promoters is shown above the transfection results for each subfigure. Each experiment was performed at least three times in duplicate and include error bars for standard deviations.

transcription.

#### **4.2.5. TAF1 Dependency of the c-Src Family Kinase Member Promoters**

Approximately 46% of human genes contain an Inr core promoter element, which is localized near or encompasses the transcription start site (Yang *et al.*, 2007). TAF1 and TAF2 are components of the TFIID multi-subunit complex which recognize and directly bind the Inr element to facilitate the assembly of the transcription pre-initiation complex (Chalkley and Verrijzer, 1999). Indeed, given this role in transcription, TAF1 has been shown to be an essential regulator of approximately 30% of genes in yeast and 18% in hamster cells (Holstege *et al.*, 1998 and O'Brien and Tjian, 2000). A *ts* hamster cell line *tsBN462*, derived from the parental BHK-21 cell line, which harbors a TAF1 mutation in the AT domain (G690A) has been an irreplaceable tool in the identification of TAF1 regulated genes, such as cyclin A and cyclin D1 (Sekiguchi *et al.*, 1996, Rushton *et al.*, 1997, Dunphy *et al.*, 2000, Hilton and Wang, 2003 and Hilton *et al.*, 2005). At a permissive temperature of 32.6°C, *tsBN462* cells exhibit normal growth and TAF1 function, while at the restrictive temperature of 39.5°C, TAF1 AT activity and Inr binding are compromised and G1/S cell cycle arrest is induced (Hayashida *et al.*, 1994, Sekiguchi *et al.*, 1996, Wang *et al.*, 1997, Dunphy *et al.*, 2000). Previously, Dr. Scott Dehm from the Bonham lab reported that the SRC1 $\alpha$  and SRC1A promoters were Inr driven and, using the *tsBN462* cell line identified that the SRC1A promoter was also dependent on TAF1 for complete promoter activity (Dehm *et al.*, 2004). In addition, TAF1 AT activity was also suggested to play a role in HDI mediated repression of SRC, as HDIs were incapable of further inhibiting the activity of the SRC1A promoter in *tsBN462* cells, at the non-permissive temperature (Dehm *et al.*, 2004). Therefore, a natural extension of this work was to identify if other SFK members also displayed a dependency on TAF1 and whether TAF1 was involved in HDI facilitated suppression of these SFKs.

Transient transfections in parental BHK-21 and matched *tsBN462* cells were carried out with 0.38SRC1A, pYSCAT2, -590LYN or -630LCK CAT reporter plasmids in the absence and presence of HDIs at the permissive (32.6°C) and restrictive temperatures (39.5°C) to confirm SRC1A TAF1 dependence and examine the other SFK member promoters (Figure 4.14.). The activity of the SRC1A promoter decreased

slightly in BHK-21 cells at the non-permissive temperature, but was suppressed by approximately 80% in *tsBN462* cells at the restrictive temperature, verifying TAF1 dependency of the SRC1A promoter (Figure 4.14.A) (Dehm *et al.*, 2004). Furthermore, TSA (1.0  $\mu$ M) was efficient at repressing the SRC1A promoter in *tsBN462* cells at the permissive temperature, but could not further prevent transcription at the restrictive temperature. Interestingly, the YES (pYSCAT2) promoter exhibited a similar pattern to that of SRC1A. The promoter activity of pYSCAT2 was downregulated approximately 30% in BHK-21 cells, while a more significant 60% reduction was observed in *tsBN462* cells at the non-permissive temperature. However, HDI mediated repression of YES did not require functional TAF1, as pYSCAT2 activity was still inhibited by TSA at the restrictive temperature in *tsBN462* cells (Figure 4.14.B). In comparison, the LYN promoter (-590LYN-CAT) itself was identified to be highly temperature sensitive, displaying an approximately 80% repression in promoter activity at the restrictive temperature in BHK-21 cells (Figure 4.14.C). Yet, the promoter activity of -590LYN-CAT was suppressed 90% in *tsBN462* cells at the non-permissive temperature, suggesting a possible role for TAF1 in LYN activation. Interestingly, the LYN promoter mimics the SRC1 $\alpha$  promoter, which was previously reported to display temperature sensitive characteristics (Dehm *et al.*, 2004). Also, it was curious to note that TSA was ineffective as an inhibitor of LYN promoter activity in both the BHK-21 and *tsBN462* cells, but has previously been effective in the SW480 cell line (Figure 4.13). TAF1 dependency of the LCK type I promoter was also investigated, but minimal LCK promoter activity was evident in BHK-21 cells (data not shown). Taken together, these results suggested that similar to the SRC1A promoter, both the YES and LYN promoters may be TAF1 dependent. However, distinct from SRC1A promoter, TAF1 does not appear to be involved in HDI mediated YES or LYN gene suppression.

#### **4.2.6. Activity of the TAF1 Acetyltransferase Domain is Required for c-Src Family Kinase Member Transcription**

Although the SRC1A promoter has been reported to display TAF1 reliance, these studies were unable to determine directly whether AT activity of TAF1 was required for SRC1A transcription, given that recombinant mutant TAF1 G690A was

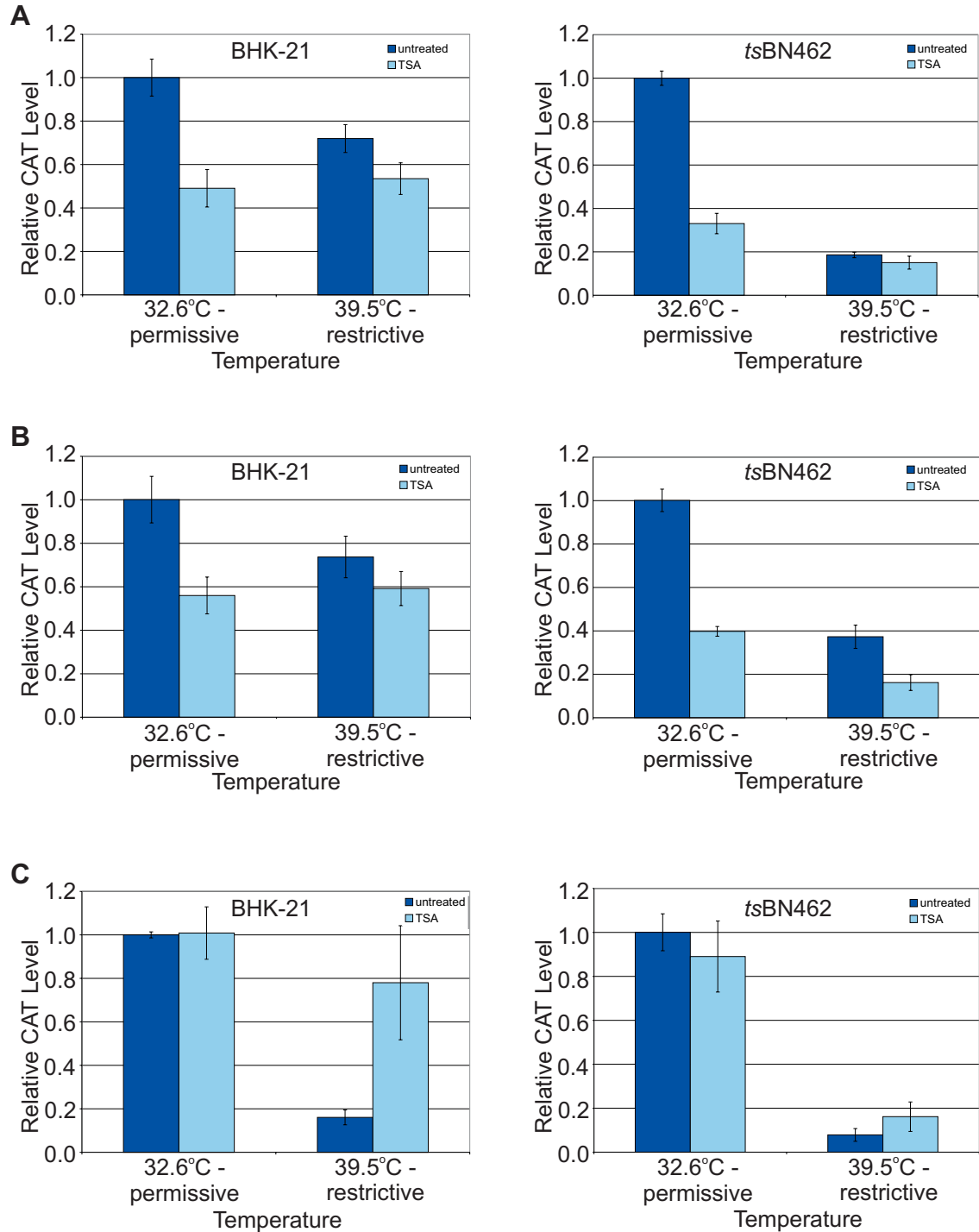


Figure 4.14. c-Src family kinase member promoters are TAF1 dependent. BHK-21 or *tsBN462* cells were transiently transfected with (A) 0.38SRC1A, (B) pYSCAT2, or (C) -590LYN-CAT and treated in the absence and presence of TSA (1.0  $\mu$ M) at the permissive or restrictive temperature. Experiments were performed a minimum of three times in duplicate and include error bars for standard deviations.

also shown to have impaired binding ability to the SRC1A Inr element at the non-permissive temperature *in vitro* (Dehm *et al.*, 2004). Therefore, SRC1A TAF1 dependency may be based solely on the ability of TAF1 to interact with the SRC1A Inr element, rather than the impairment of TAF1 AT activity. To address the role of TAF1 AT activity in transcription of the SRC1A promoter, a series of mutant TAF1 AT domain expression plasmids were obtained from a collaborator, Dr. Edith Wang, at the University of Washington: TAF1 $\Delta$ 574-590, TAF1 $\Delta$ 844-850 ( $\Delta$ Stu), TAF1 $\Delta$ 848-850 ( $\Delta$ MED) and TAF1G923/925D (Figure 4.15.) (Hilton *et al.*, 2005). The TAF1 $\Delta$ 574-590 mutant contains a 16 amino acid deletion in the TAF1 AT domain, but the TAF1 AT activity remains intact. Mutants TAF1 $\Delta$ 848-850 ( $\Delta$ Stu) and TAF1 $\Delta$ 844-850 ( $\Delta$ MED) both exhibit severely compromised TAF1 AT activity. Finally, the TAF1G923/925D double mutant possesses AT activity comparable to wild-type TAF1 and was utilized as an experimental control.

Transient co-transfections were performed in *tsBN462* cells with the 0.38SRC1A-CAT reported plasmid and wild-type (wt) TAF1, TAF1G923/925D, TAF1 $\Delta$ 574-590 TAF1 $\Delta$ Stu, or TAF1 $\Delta$ MED expression vectors to establish which TAF1 mutants could restore the SRC1A promoter activity at the restrictive temperature (Figure 4.16.A). As previously reported, promoter activity of SRC1A can be rescued by wt TAF1 (Dehm *et al.*, 2004). Remarkably, TAF1G923/925D and TAF1 $\Delta$ 574-590 mutants also effectively reinstated SRC1A activity, however, neither the TAF1 $\Delta$ Stu nor TAF1 $\Delta$ MED mutants reconstituted SRC1A promoter activity at the non-permissive temperature. In addition, TAF1 dependency and the importance of TAF1 AT activity in YES and LYN promoter activation were studied. Activation of the YES promoter was partially restored by the TAF1 wt, TAF1G923/925D and TAF1 $\Delta$ 574-590 expression plasmids, but once again TAF1 $\Delta$ Stu and TAF1 $\Delta$ MED mutants were deficient at rescuing YES promoter activity in *tsBN462* cells at the restrictive temperature (Figure 4.16.B). Furthermore, TAF1 wt and TAF1G923/925D and TAF1 $\Delta$ 574-590 vectors marginally rescued LYN promoter activity at the restrictive temperature, while the TAF1 $\Delta$ Stu and TAF1 $\Delta$ MED plasmids were entirely incompetent at reactivating the LYN promoter. Therefore, these observations further imply that the YES and LYN

**A**

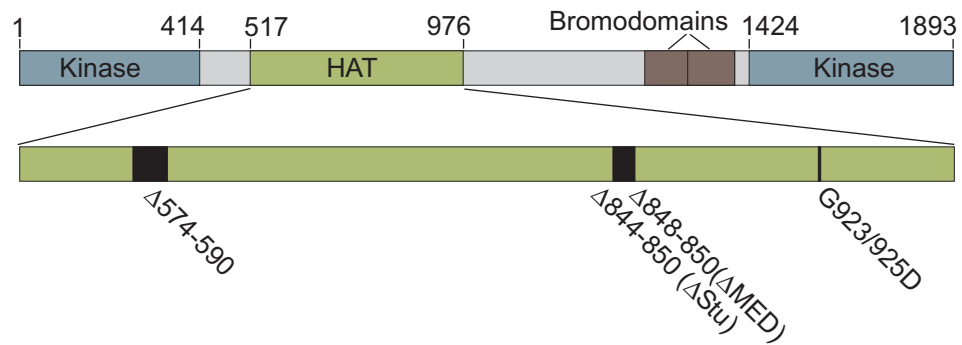


Figure 4.15. Structural Domains of TAF1. The N-terminal and C-terminal kinase domains are shown along with the bromodomains and acetyltransferase (AT) domain. The AT domain is enlarged to highlight the positions of TAF1 AT deletions and mutations.

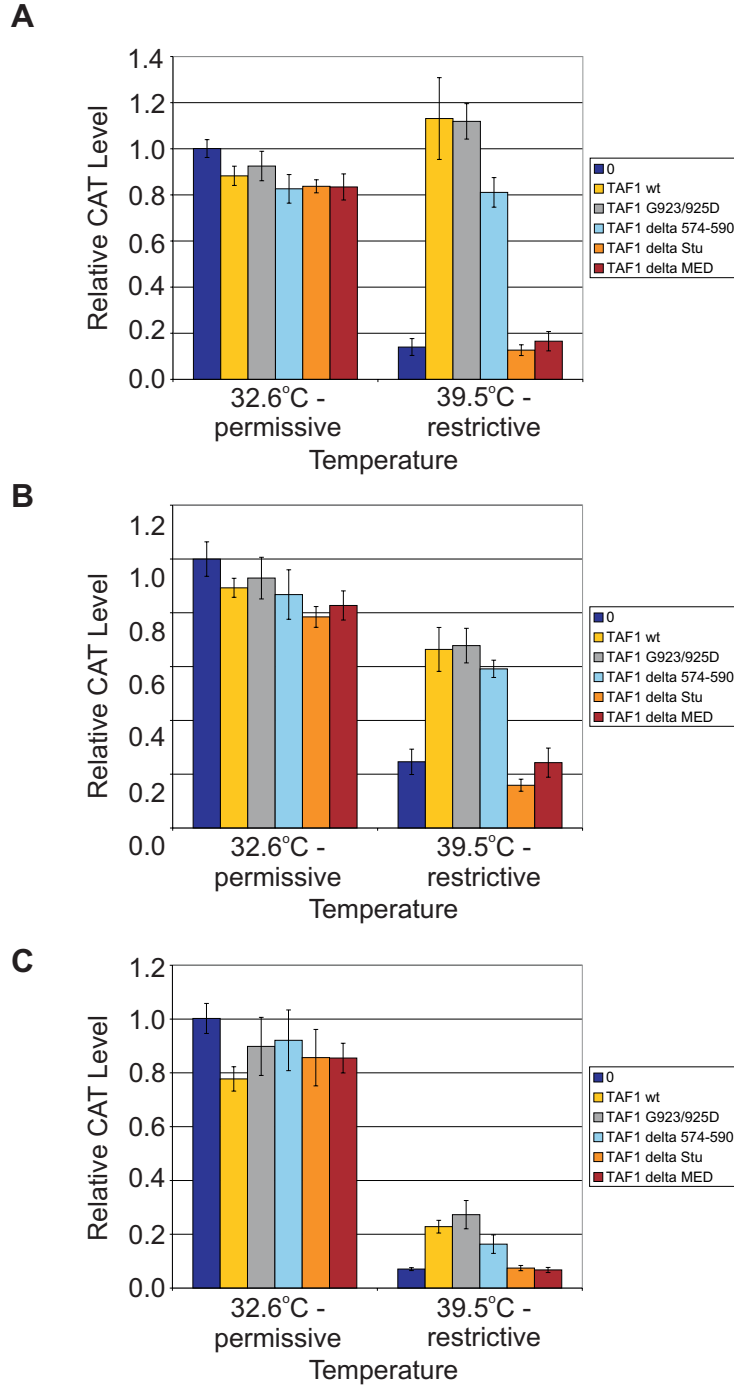


Figure 4.16. TAF1 acetyltransferase activity is required to restore SFK promoter activity. Transient co-transfections were performed in *tsBN462* cells with wt or various TAF1 AT deletion and mutant expression vectors in the presence of (A) 0.38SRC1A, (B) pYSCAT2, or (C) -590LYN-CAT at the permissive and restrictive temperatures. All experiments were repeated twice in duplicate and include error bars for standard deviations.



promoters are TAF1 dependent and suggest that SRC1A, YES and LYN basal transcription requires TAF1 AT activity.

#### **4.2.7. SUMMARY**

c-Src and a number of the SFK members have been shown to play a role in colon neoplasias (Biscardi et al., 1999, Frame, 2002, Russello and Shore, 2003 and Summy and Gallick, 2003). Prior studies demonstrated that HDIs downregulates c-Src expression in human colon, breast and hepatocellular carcinoma cells (Kostyniuk *et al.*, 2002). Therefore, given that SFK members are exceedingly alike and have been suggested to compensate for one another, the response of SFK members to HDIs, in particular butyrate, was undertaken (Lowell and Soriano, 1996 and Stein *et al.*, 1994). First, the relative expression patterns of SFKs were investigated in a panel of HCCLs and it was determined that most of these cell lines expressed relatively high levels of at least two SFKs. Furthermore, when the effects of NaB were monitored on SFK members in HCCLs, downregulation of the SFK members was observed following 24 h of NaB (5.0 mM) or TSA (1.0  $\mu$ M) exposure. Similar to c-Src, NaB directly repressed Lck and Lyn mRNA expression levels, independent of new protein synthesis. However, HDI mediated inhibition of Yes gene expression could be either directly or indirectly mediated by HDIs, as these results were inconclusive. HDIs were sufficient inhibitors of LCK type I, LYN and YES promoter activities. In addition, the YES and LYN promoters were characterized to be TAF1 dependent, and TAF1 AT was determined to be a requirement for SRC1A, YES and LYN basal transcription activity. Impairment of TAF1 AT activity did not, therefore, prevent HDI facilitated suppression of YES. Taken together these results suggest that HDIs may globally inhibit SFKs (Hirsch *et al.*, 2006).

### **4.3. HISTONE DEACETYLASE INHIBITOR REGULATION OF p21WAF1 IN HEPG2 CELLS**

#### **4.3.1. New Protein Synthesis is Required for HDI Induction of p21WAF1 in HepG2 cells**

HDI mediated activation of p21WAF1 has been intimately linked to cellular G1/S growth arrest, and reported to be a necessary factor in HDI prevention of tumor cell growth (Archer *et al.*, 1998, Rosato *et al.*, 2001 and Rosato *et al.*, 2003). A number of previous reports have demonstrated that HDIs stimulate p21WAF1 gene expression independent of p53 and protein neo-synthesis (Nakano *et al.*, 1997, Sowa *et al.*, 1997 and Huang *et al.*, 2000). Furthermore, HDIs have been documented to induce histone hyperacetylation of the p21WAF1 gene promoter and downstream nucleosomes, and to activate the promoter primarily through the third Sp site (Nakano *et al.*, 1997 and Sowa *et al.*, 1997, Saito *et al.*, 1999, Han *et al.*, 2000 Richon *et al.*, 2000, Han *et al.*, 2001 and Kim *et al.*, 2001). However, material presented in the first results section of this thesis noted a surprising observation; HDIs required new protein synthesis to upregulate p21WAF1 mRNA expression levels in HepG2 cells (Figure 4.7.D) (shown again in Figure 4.17.A). Given these curious data, the effects of HDIs on p21WAF1 gene expression were more thoroughly studied in HepG2 cells.

Since NaB (5.0 mM) enhanced p21WAF1 mRNA expression levels in HepG2 cells in the absence of the protein synthesis inhibitor CHX (50  $\mu$ M), but not in its presence, time course studies were performed with HepG2 cells exposed to either NaB (5.0 mM) or TSA (1.0  $\mu$ M) to assess p21WAF1 protein levels. In agreement with p21WAF1 mRNA levels, Western blots demonstrated a dramatic time dependent increase in p21WAF1 protein expression, cumulatively suggesting that HDIs indirectly upregulated p21WAF1 mRNA in HepG2 cells (Figure 4.17.B, C). Furthermore, basal p21WAF1 expression levels were detectable in HepG2 cells, unlike most cell lines.

#### **4.3.2. Histone Deacetylase Inhibitors Cannot Activate the p21WAF1 Promoter in HepG2 Cells**

Since NaB stimulation of p21WAF1 gene expression appears to be dependent on *de novo* protein synthesis, the ability of HDIs to indirectly activate the p21WAF1

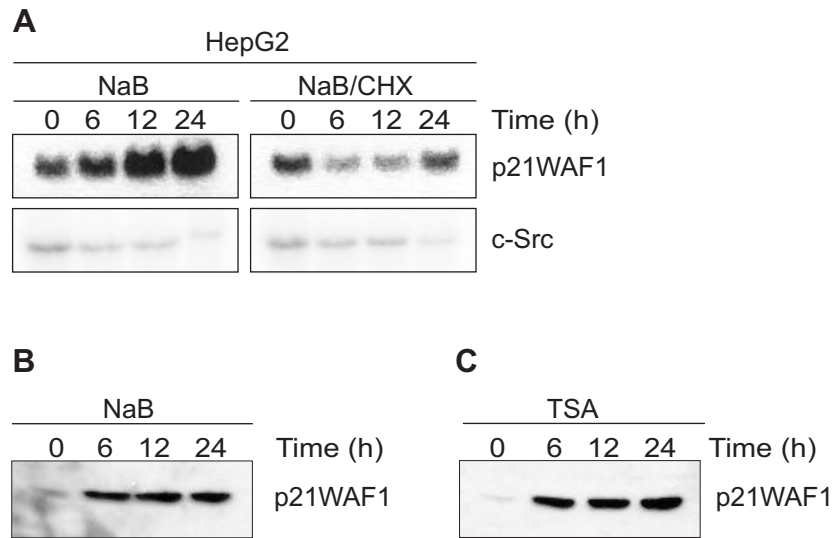


Figure 4.17. Histone deacetylase inhibitor upregulation of p21WAF1 gene expression in HepG2 cells requires protein neo synthesis. A. Total RNA was extracted from HepG2 cells following various periods of exposure to NaB (5.0 mM), CHX (50  $\mu$ M), or a combination of the two. RNA was examined by Northern blot analysis for p21WAF1 and c-Src. B, C. Total cellular extracts were isolated from HepG2 cells following incubation with NaB (5.0 mM) or TSA (1.0  $\mu$ M) for varying time points and analyzed by Western blot for p21WAF1 protein expression. Representative Northern and Western blots of three separate experiments are shown.

promoter in HepG2 cells was monitored. Transient transfection assays were performed in HepG2 cells with WAF1-CAT reporter plasmids in the absence or presence of HDIs. Two different WAF1-CAT promoter constructs that have previously been shown to be inducible by HDIs were utilized to determine if the WAF1 promoter could be activated by HDIs in HepG2 cells (Nakano *et al.*, 1997, Archer *et al.*, 1998 and Han *et al.*, 2001). HT29 or HepG2 cells were transiently transfected with -210WAF1 or -101WAF1 CAT promoter constructs and treated with either NaB (5.0 mM) or TSA (1.0  $\mu$ M) (Figure 4.18.). Transient transfections performed in HT29 cells verified that the WAF1 promoter constructs were activated as expected (Figure 4.18. B). In these cells, the promoter activity of -210WAF1 CAT was increased 15 and 30-fold by NaB and TSA, respectively. Similarly, the -101WAF1 CAT promoter construct was also activated approximately 6 and 4-fold by NaB and TSA, respectively. However, in HepG2 cells neither the -210WAF1 and -101WAF1 promoters were activated by TSA, and were only marginally upregulated by NaB (Figure 4.18.C). The larger -2300WAF1 CAT reporter plasmid, which harbors two p53 REs was similarly unresponsive to HDIs in HepG2 cells (results not shown). Since p21WAF1 is constitutively expressed at relatively high levels in HepG2 cells, it was important to determine if the WAF1 promoter could realistically benefit from supplemental transcriptional activation. Co-transfection experiments were therefore performed with the WAF1 promoter vectors in the presence of c-jun, a known p21WAF1 trans-activator (Kardassis *et al.*, 1999). Co-transfection data confirmed that the WAF1 promoter was capable of further activation, being upregulated by c-jun in HepG2 cells approximately 2.5-fold and 6.0-fold in -210WAF1 CAT and -101WAF1 CAT activity, respectively (Figure 4.18.C). These observations indicated that the p21WAF1 promoter is unresponsive to HDIs, and suggested that HDIs induce p21WAF1 gene expression by post-transcriptional mechanisms in HepG2 cells.

#### **4.3.3 p21WAF1 mRNA is Regulated Post-transcriptionally by Histone Deacetylase Inhibitors**

A series of mRNA half-life studies were executed in HepG2 cells. Actinomycin D (Act D) was added to HepG2 cells to prevent transcription after a 12 h incubation

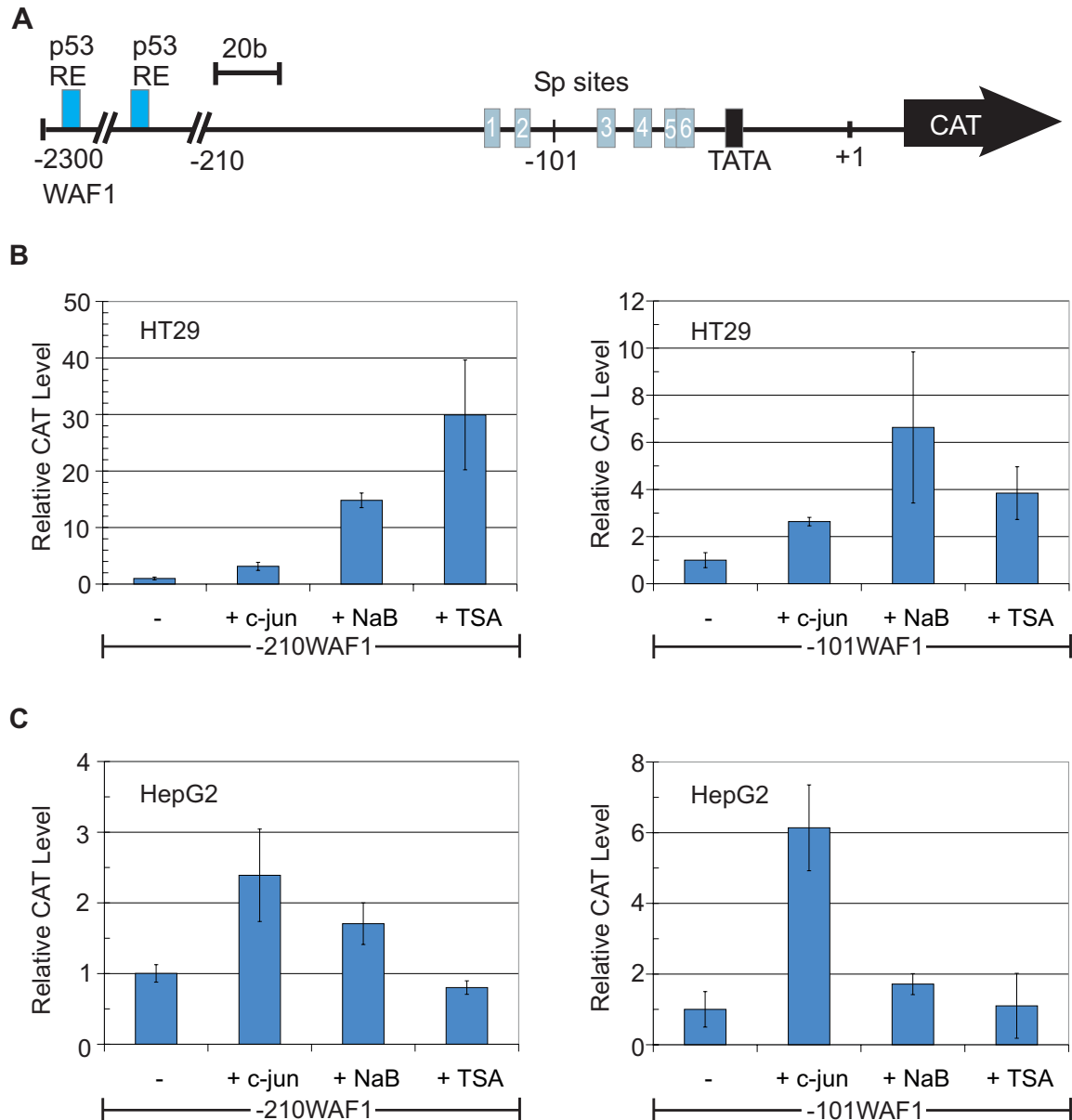


Figure 4.18. The WAF1 promoter is not activated by histone deacetylase inhibitors in HepG2 cells. A. Diagram representing various WAF1-CAT constructs used in this study, showing the positions of critical Sp sites. HT29 (B) or HepG2 (C) cells were transiently transfected with WAF1-CAT promoter constructs; -210WAF1-CAT and -101WAF1-CAT. B, C. Co-transfections were performed with the WAF1 promoter constructs and exposed to NaB (5.0 mM), TSA (1.0  $\mu$ M), or left untreated. A second set of co-transfections were performed with the WAF1 promoter constructs and c-jun. The CAT levels were determined relative to the untreated WAF1 co-transfected HT29 (B) and HepG2 cells (C). Results represent two independent experiments performed in duplicate and error bars denote the standard deviations.

period in the presence or absence of either, NaB (5.0 mM) or TSA (1.0  $\mu$ M). Northern blot analysis was then performed to examine p21WAF1 mRNA levels (Figure 4.19.A). Ribosomal Protein PO (RPPO) was used as a control to normalize the data. The half-life of p21WAF1 mRNA in untreated HepG2 cells was calculated to be approximately 85 min. However, in HepG2 cells exposed to either NaB or TSA the p21WAF1 mRNA half-life increased approximately 3.1-fold (270min) and 5.4-fold (460min), respectively (Figure 4.19.B). Taken together, these data show that NaB and TSA upregulate p21WAF1 mRNA and protein levels by post-transcriptional mechanisms in HepG2 cells. These results represented the first reported example of HDI mediated mRNA stabilization (Hirsch and Bonham, 2004).

#### **4.3.4. The Role of the p21WAF1 3' Untranslated Region in Histone Deacetylase Inhibitor Mediated Stabilization of p21WAF1 mRNA**

Post-transcriptional regulation is an important regulatory mechanism in the control of gene expression. mRNA levels can be stabilized by numerous mechanisms, including the association of *trans*-RNA binding proteins with *cis*-acting elements in the 3' UTR of many mRNAs (Ross, 1995). One of the most well defined 3' UTR *cis*-acting stability determinants is the ARE. AREs are inherently destabilizing elements that mediate deadenylation of the mRNA poly (A) tail (Chen and Shyu, 1995). The interaction of *trans*-associated factors such as AUF1 and members of the ELAV and TTP families with AREs may either enhance or suppress mRNA decay rates (Barreau *et al.*, 2005 and Lai *et al.*, 2006). The 3' UTR of the p21WAF1 mRNA contains an ELAV binding element recognized by HuD, and three well characterized AREs (A, B and C) that have been documented to mediate UVC-induced stabilization of p21WAF1 mRNA levels (Figure 4.20.) (Figure 4.21.A) (el-Deiry *et al.*, 1993, Joseph *et al.*, 1998 and Wang *et al.*, 2000). Given that new protein synthesis is necessary for HDI mediated upregulation of p21WAF1 gene expression levels, the interaction of key *cis*-acting p21WAF1 3' UTR mRNA stability determinants with a newly synthesized *trans*-associated RNA binding protein was hypothesized to alter the p21WAF1 mRNA decay rate in HepG2 cells exposed to HDIs.

To study this hypothesis, a transient transfection system was employed to look

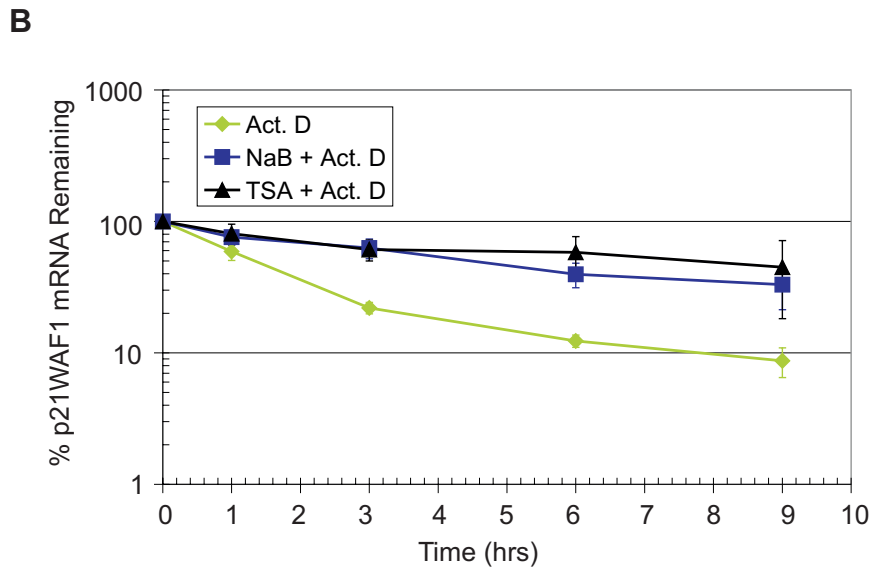
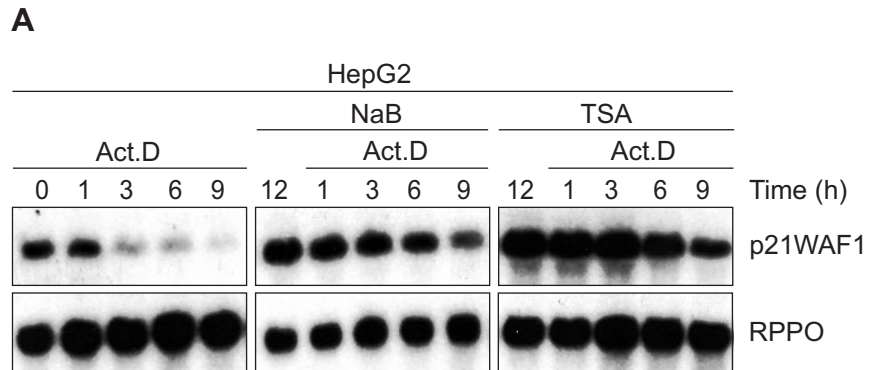


Figure 4.19. Histone deacetylase inhibitors increase the stability of p21WAF1 mRNA in HepG2 cells. A. Total RNA was isolated from HepG2 cells treated with Act. D in the presence or absence of NaB (5.0 mM) or TSA (1.0  $\mu$ M) and examined by Northern blot analysis for p21WAF1 and RPPO. B. The p21WAF1 mRNA signal was determined by a Molecular Imager and normalized to the RPPO mRNA signal. Data were plotted on a semi-logarithmic scale and represent means  $\pm$  the standard errors of two independent experiments.

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571  TCCGCCACACA GGAAGCCTGC AGTCCTGGAA GCGCGAGGGC CTCAAAGGCC CGCTCTACAT
631  CTTCTGCCTT AGTCTCAGTT TGTGTGTCTT AATTATTATT TGTGTTTTAA TTAAACACC
                                           HuD
691  TCCTCATGTA CATAACCTGG CCGCCCCCTG CCCCCCAGCC TCTGGCATTGA GAATTATTTA
                                           ARE (A)
751  AACAAAAACT AGGCGGTTGA ATGAGAGGTT CCTAAGAGTG CTGGGCATTT TTATTTTATG
                                           ARE (B)
811  AAATACTATT TAAAGCCTCC TCATCCCGTG TTCTCCTTTT CCTCTCTCCC GGAGGTTGGG
                                           ARE (C)
871  TGGGCCCGGCT TCATGCCAGC TACTTCCTCC TCCCCACTTG TCCGCTGGGT GGTACCCTCT
931  GGAGGGGTGT GGCTCCTTCC CATCGCTGTC ACAGGCGGTT ATGAAATTCA CCCCCTTTCC
991  TGGACACTCA GACCTGAATT CTTTTTCATT TGAGAAGTAA ACAGATGGCA CTTTGAAGGG
1051 GCCTCACCGA GTGGGGGCAT CATCAAAAAC TTTGGAGTCC CCTCACCTCC TCTAAGGTTG
1111 GGCAGGGTGA CCCTGAAGTG AGCACAGCCT AGGGCTGAGC TGGGGACCTG GTACCCTCCT
1171 GGCTCTTGAT ACCCCCCTCT GTCTTGTGAA GGCAGGGGGA AGGTGGGGTA CTGGAGCAGA
1231 CCACCCCGCC TGCCCTCATG GCCCCTCTGA CCTGCACTGG GGAGCCCGTC TCAGTGTTGA
1291 GCCTTTTCCC TCTTTGGCTC CCCTGTACCT TTTGAGGAGC CCCAGCTTAC CCTTCTTCTC
1351 CAGCTGGGCT CTGCAATTCC CCTCTGCTGC TGTCCCTCCC CCTTGTCTTT CCCTTCAGTA
1411 CCCTCTCATG CTCCAGGTGG CTCTGAGGTG CCTGTCCCAC CCCCACCCCC AGCTCAATGG
1471 ACTGGAAGGG GAAGGGACAC ACAAGAAGAA GGGCACCCCTA GTTCTACCTC AGGCAGCTCA
1531 AGCAGCGACC GCCCCCTCCT CTAGCTGTGG GGGTGAGGGT CCCATGTGGT GGCACAGGCC
1591 CCCTTGAGTG GGGTTATCTC TGTGTTAGGG GTATATGATG GGGGAGTAGA TCTTTCTAGG
1651 AGGGAGACAC TGGCCCCCTCA AATCGTCCAG CGACCTTCCT CATCCACCCC ATCCCTCCCC
1711 AGTTCATTGC ACTTTGATTA GCAGCGGAAC AAGGAGTCAG ACATTTTAAG ATGGTGGCAG
1771 TAGAGGCTAT GGACAGGGCA TGCCACGTGG GCTCATATGG GGCTGGGAGT AGTTGTCTTT
1831 CCTGGCACTA ACGTTGAGCC CCTGGAGGCA CTGAAGTGCT TAGTGTACTT GGAGTATTGG
1891 GGTCTGACCC CAAACACCTT CCAGCTCCTG TAACATACTG GCCTGGACTG TTTTCTCTCG
1951 GCTCCCCATG TGTCTGGTT CCCGTTTCTC CACCTAGACT GTAAACCTCT CGAGGGCAGG
2011 GACCACACCC TGTACTGTTT TGTGTCTTTC ACAGCTCCTC CCACAATGCT GAATATACAG
2071 CAGGTGCTCAA ATAAATGATT CTTAGTGACT TT
                                           poly (A) site hexamer

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Figure 4.20. The sequence of the p21WAF1 3' untranslated region. The 3' UTR corresponds to nucleotides 571-2102 of the p21WAF1 mRNA. The sequence of the HuD site and AREs (A-C) are underlined and shown in blue. The poly (A) site hexamer is highlighted in red.



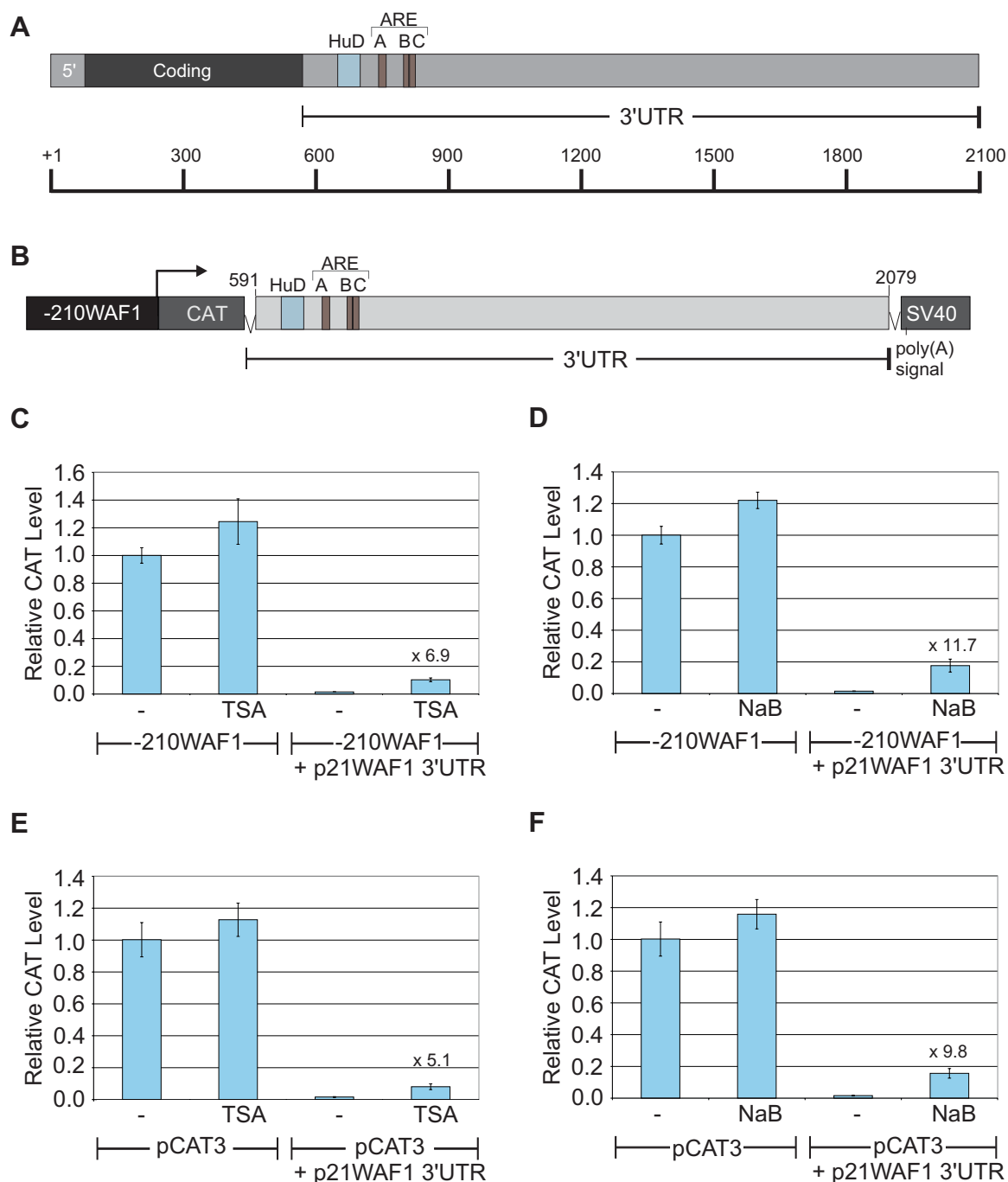


Figure 4.21. The p21WAF1 3' UTR plays a role in histone deacetylase inhibitor mediated mRNA stabilization in HepG2 cells. (A) The p21WAF1 mRNA is shown containing the 5' UTR, coding region and 3' UTR. Key cis acting 3' UTR elements are denoted by the HuD element and three AU-rich elements (AREs - A, B, C). (B) A cartoon of the -210WAF1-CAT + p21WAF1 3'UTR 591-2079 (FL) vector is displayed. -210WAF1-CAT + p21WAF1 3'UTR (FL) and empty -210WAF1CAT vectors were transiently transfected in HepG2 cells treated in the absence or presence of TSA (1.0  $\mu$ M) (C) or NaB (5.0 mM) (D). An empty SV40 driven pCAT3 promoter vector or a pCAT3 promoter vector + p21WAF1 3'UTR (FL) were transiently transfected and treated as described above (E, F). Experiments were performed twice in duplicate.

for relevant p21WAF1 mRNA *cis*-acting stability elements influenced by HDIs. First, an appropriate vector was constructed. The p21WAF1 mRNA 3'UTR 591-2079, lacking the original p21WAF1 poly(A) signal hexamer motif AATAAA, was cloned into the -210WAF1 CAT promoter construct downstream of the CAT reporter but upstream of the vector's SV40 late poly(A) region and signal to generate a -210WAF1-CAT + p21WAF1 3'UTR 591-2079 (FL) plasmid (Figure 4.21.B). Transient transfections were performed with the -210WAF1-CAT or -210WAF1-CAT + p21WAF1 3'UTR (FL) vector in the absence and presence of TSA in HepG2 cells and CAT levels determined to assess whether addition of the p21WAF1 3'UTR influenced mRNA stability and subsequent CAT synthesis. Interestingly, addition of the p21WAF1 3' UTR significantly decreased basal activity of the -210WAF1-CAT + p21WAF1 3'UTR (FL) plasmid compared to -210WAF1-CAT transfected cells (Figure 4.21.C, D). Furthermore, the -210WAF1-CAT vector was not significantly upregulated by TSA (1.0  $\mu$ M) nor NaB (5.0 mM), while the -210WAF1-CAT + p21WAF1 3' UTR (FL) plasmid exhibited an elevation in CAT activity following TSA and NaB treatment in HepG2 cells: 6.9 and 11.7- fold, respectively. As a control, the p21WAF1 3'UTR (FL) was also cloned into a pCAT3 promoter vector downstream of a SV40 promoter and upstream of the SV40 late poly(A) region and signal to generate a pCAT3 + p21WAF1 3'UTR (FL) plasmid (vector not shown). Similar to the initial observations, neither TSA nor NaB could activate the SV40 driven pCAT3 promoter vector (Figure 4.21.E, F). However, the presence of the p21WAF1 3'UTR, downstream of the SV40 promoter in the pCAT3 vector increased HDI responsiveness in HepG2 cells. Therefore, these observations suggested that the p21WAF1 3' UTR plays an important role in HDI mediated p21WAF1 mRNA stabilization, even out of the context of its own promoter. Furthermore, transient transfections of -210WAF1-CAT + p21WAF1 3'UTR vectors, followed by CAT assays, appeared to be an effective method to monitor the status of p21WAF1 mRNA stability. The -210WAF1-CAT vector provided the ideal backdrop for these experiments as this promoter was shown to be relatively non-responsive to HDIs, sequence from the -210WAF1 promoter did not indirectly influence p21WAF1 mRNA stability levels and the p21WAF1 promoter was allowed to be paired with its 3' UTR adding a level of physiological relevance to these experiments.

#### 4.3.4.1. Proximal p21WAF1 3'Untranslated Region Deletions

To further assess the role of the p21WAF1 mRNA 3' UTR in HDI induction of mRNA stability, it was sought to identify *cis*-acting elements within the 3' UTR that may be responsible for this effect. Deletions were made in the proximal region of the p21WAF1 3' UTR, given the presence of well characterized destabilizing elements within the 591-840 region (el-Deiry *et al.*, 1993, and Joseph *et al.*, 1998). Four vectors were generated exactly as described in the previous section, where a deleted version of the p21WAF1 3' UTR was cloned downstream of the -210WAF1-CAT, but upstream of the SV40 late poly(A) region and signal: -210WAF1-CAT + p21WAF1 3'UTR 712-2079 ( $\Delta$ HuD), -210WAF1-CAT + p21WAF1 3'UTR 788-2079 ( $\Delta$ HuD/ $\Delta$ ARE(A)), -210WAF1-CAT + p21WAF1 3'UTR 840-2079 ( $\Delta$ HuD/ $\Delta$ ARE(sites)), and -210WAF1-CAT + p21WAF1 3'UTR 591-840 (Figure 4.22.). HepG2 cells were transiently transfected with these deletion plasmids individually and the basal CAT activity of the -210WAF1-CAT + p21WAF1 3'UTR deletion vectors compared, relative to that of the -210WAF1-CAT vector lacking a p21WAF1 3'UTR (Figure 4.23.). Surprisingly, all of the deletion plasmids examined lacking the HuD site and AREs between 591-840 of the p21WAF1 3'UTR had destabilizing potential, compared to the reporter plasmid containing only the p21WAF1 3'UTR 591-840 region (Figure 4.23.A). These results are a direct contradiction to numerous reports stating that AREs boost mRNA decay and suggest that the nucleotides encompassing 840-2079 of the 3' UTR contain a powerful, and yet uncharacterized, destabilizing element.

In addition to elucidating the relative CAT levels of the -210WAF1-CAT + p21WAF1 3'UTR deletion plasmids, the ability of TSA (1.0  $\mu$ M) and NaB (5.0 mM) to mediate p21WAF1 mRNA stability was examined (Figure 4.23.B, C). The data revealed that p21WAF1 mRNA levels remained stabilized upon deletion of the HuD site and AREs, following exposure to HDIs. However, HDIs failed to stabilize the -210WAF1-CAT + p21WAF1 3'UTR (591-840) deletion vector, indicating that the nucleotide sequence between 840-2079 of the p21WAF1 3' UTR may harbor an important *cis*-acting element that is required to mediate HDI stabilization of p21WAF1 mRNA levels.

-210WAF1-CAT + p21WAF1 3'UTR 591-2079 (FL)

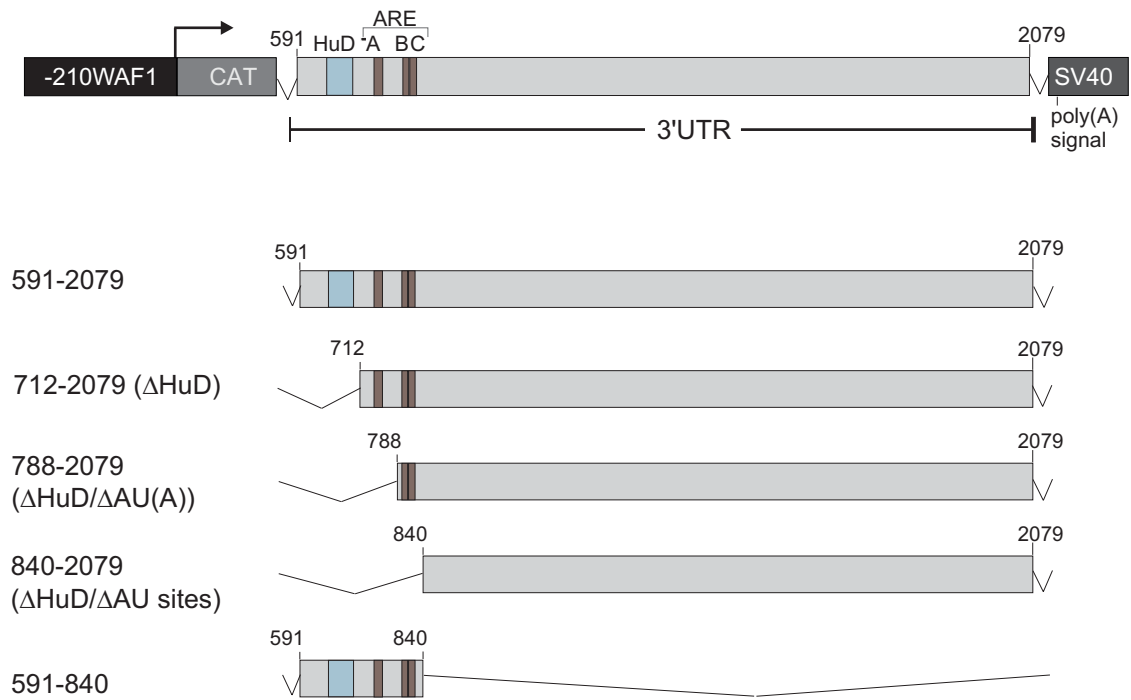


Figure 4.22. The -210WAF1-CAT + p21WAF1 3'UTR proximal deletion constructs. The p21WAF1 3'UTRs were cloned downstream of the -210WAF1 promoter and CAT coding region, but upstream of the late SV40 poly(A) region and signal as shown for the 591-2079 (FL) vector. 5' deletions of the p21WAF1 3' UTR that were cloned into the -210WAF1-CAT vector are shown.

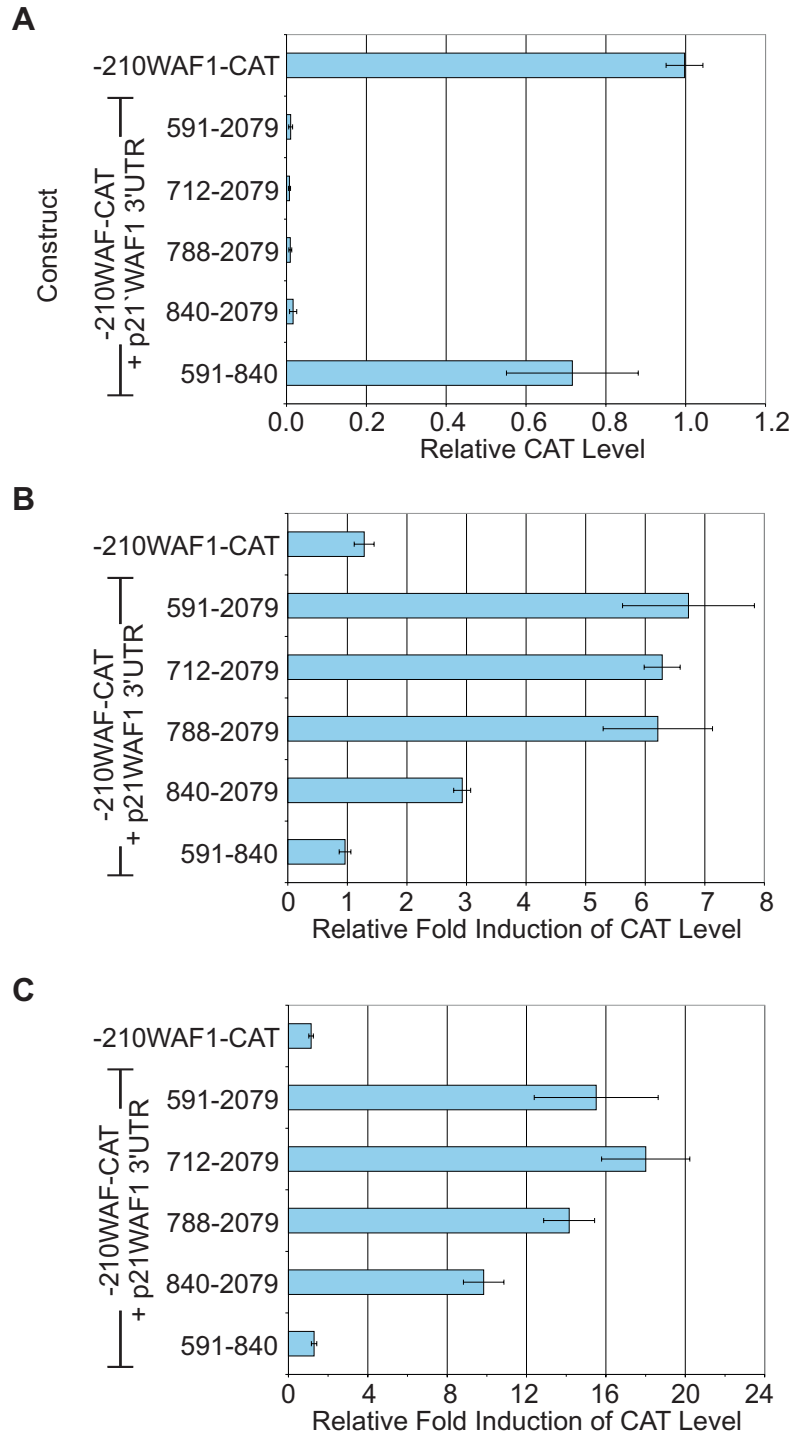


Figure 4.23. The p21WAF1 3' UTR, HuD and AU-rich elements do not play a role in histone deacetylase inhibitor mediated p21WAF1 mRNA stabilization. (A) HepG2 cells were transiently transfected with empty -210WAF1-CAT or -210WAF1-CAT + p21WAF1 3'UTR deletion vectors and basal CAT levels were used to determine mRNA stabilization relative to -210WAF1-CAT. Transfections were also carried out in the absence and presence of TSA (1.0  $\mu$ M) (B) or NaB (5.0 mM) (C). CAT values are represented as fold induction relative to untreated cells. The results represent two independent experiments performed in duplicate. Standard deviations are included as error bars.

#### 4.3.4.2. Further 5' Deletions of the p21WAF1 3' Untranslated Region

A second set of p21WAF1 3' UTR deletion vectors were constructed, as discussed above, to focus on the nucleotides between 840-1272 of the p21WAF1 3'UTR. Five deletion plasmids were created: -210WAF1-CAT + p21WAF1 3'UTR 932-2079, 210WAF1-CAT + p21WAF1 3'UTR 1045-2079, 210WAF1-CAT + p21WAF1 3'UTR 1173-2079, 210WAF1-CAT + p21WAF1 3'UTR 1272-2079, and -210WAF1-CAT + p21WAF1 3'UTR 840-1470 (Figure 4.24.). CAT levels of these deletion vectors were directly compared to those of -210WAF1-CAT, and results suggested that an instability determinant existed between nucleotides 1045-1272 of the p21WAF1 mRNA 3' UTR (Figure 4.25.A). Furthermore, p21WAF1 mRNA was radically stabilized by TSA (1.0  $\mu$ M) and NaB (5.0 mM) in cells transiently transfected with the full length -210WAF1-CAT + p21WAF1 3'UTR (591-2079) or truncated 932-2079 p21WAF1 3'UTR vectors (Figure 4.25.B, C). However, HDIs were impaired in their ability to stabilize p21WAF1 mRNA in cells transfected with -210WAF1-CAT + p21WAF1 3'UTR 1045-2079, p21WAF1 3'UTR 1173-2079 and, in particular, p21WAF1 3'UTR 1272-2079. These results suggested that the p21WAF1 mRNA contains a *cis*-acting HDI responsive element between nucleotides 932-1272 of the 3' UTR. Although when the -210WAF1-CAT + p21WAF1 3'UTR 840-1470 vector was transfected into HepG2 cells, mRNA stabilization could also not be restored in the presence of TSA or NaB. However, while TSA could not elevate the CAT level of the -210WAF1-CAT + p21WAF1 3'UTR 840-1470 vector, CAT levels were enhanced 3-fold by NaB, suggesting that pleiotropic effects, rather than inhibition of HDACs, may influence NaB mediated mRNA stabilization of p21WAF1. Taken together, these data implicate a potential instability determinant between 1045-2079 of the 3' UTR. Yet an additional *cis*-acting instability element clearly resides within nucleotides 1272-2079 of the p21WAF1 3' UTR mRNA species. In addition, a *cis*-acting HDI responsive element may be located within the 932-1272 region of the p21WAF1 3'UTR, although this element likely cooperates with another *cis*-acting element within the 1470-2079 region of the p21WAF1 3'UTR to effectively stabilize mRNA levels.

-210WAF1-CAT + p21WAF1 3'UTR 591-2079 (FL)

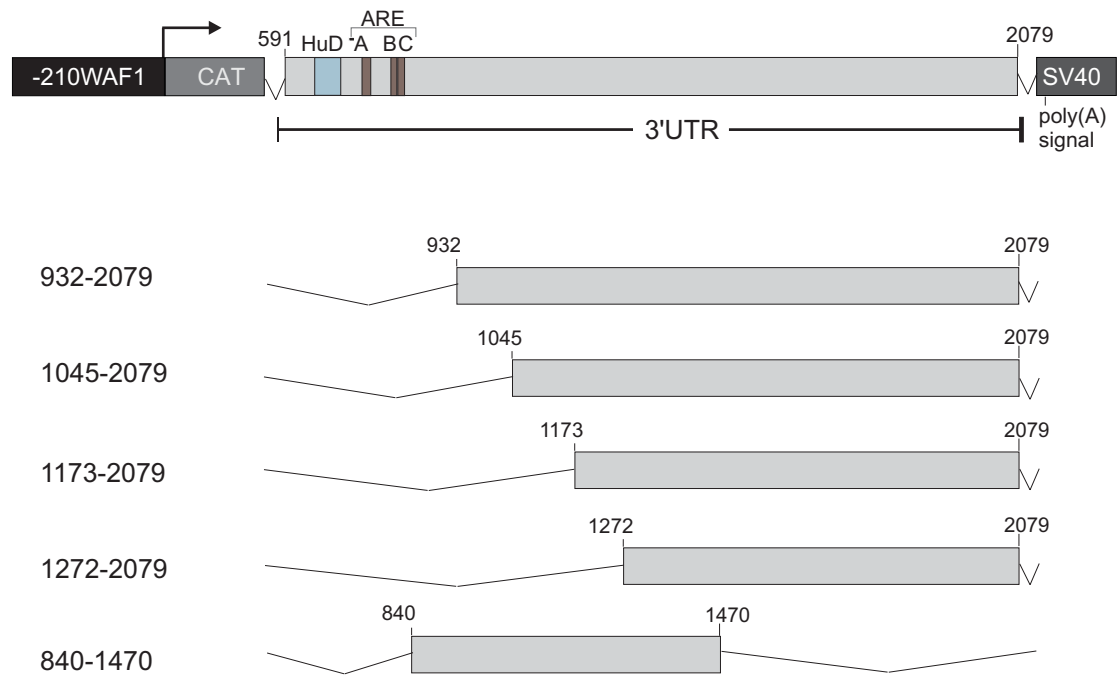


Figure 4.24. Further -210WAF1-CAT + p21WAF1 3'UTR 5' deletions. The highlighted p21WAF1 3'UTRs were cloned into the -210WAF1-CAT vector downstream of the CAT coding region and upstream of the late SV40 poly(A) region and signal, as illustrated for the 591-2079 (FL) vector.

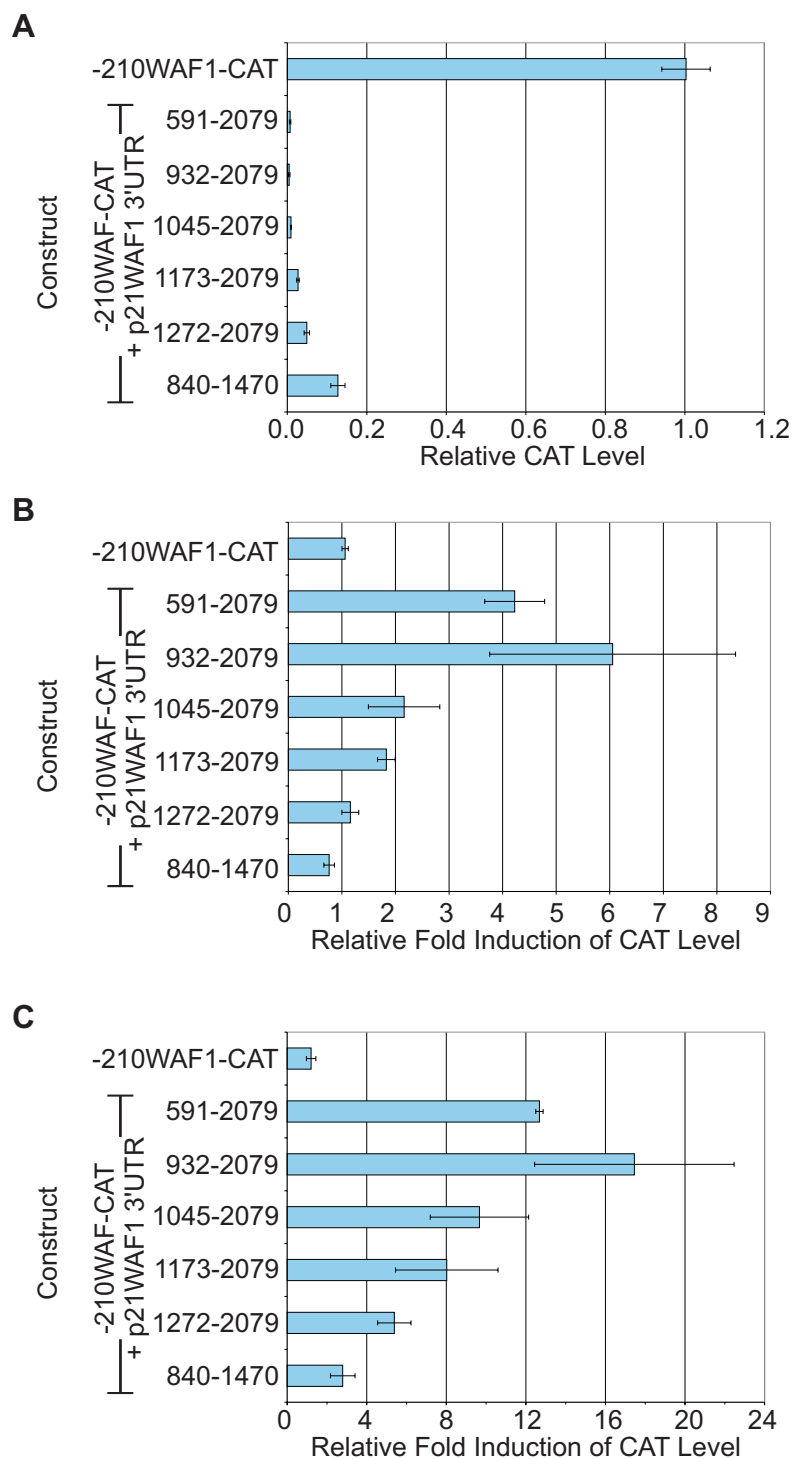


Figure 4.25. 5' Deletions of the p21WAF1 3' UTR highlight a potential *cis*-acting sequence. (A) Transient transfections were performed in HepG2 cells with empty -210WAF1-CAT or -210WAF1-CAT + p21WAF1 3'UTR deletion vectors and the relative fold induction of CAT levels was determined. Transfected cells treated with TSA (1.0  $\mu$ M) (B) or NaB (5.0 mM) (C) are represented as fold induction relative to untreated self. Average values of at least two transfections performed in duplicate are shown and standard deviations are included as error bars.



#### **4.3.4.3. Deletions of the p21WAF1 3' Untranslated Region at the Extreme 3' End**

In an attempt to search for other *cis*-acting instability determinants and HDI responsive elements within the p21WAF1 3' UTR, an additional series of vectors were produced that contained 3' deletions. The plasmids -210WAF1 + p21WAF1 3'UTR 591-1470, -210WAF1 + p21WAF1 3'UTR 591-1777, -210WAF1 + p21WAF1 3'UTR 591-1885, and -210WAF1 + p21WAF1 3'UTR 591-1779 were created. Interestingly, when transfected into HepG2 cells, each of these plasmids displayed similar basal CAT levels, approximately 5-fold higher than full length -210WAF1 + p21WAF1 3'UTR 591-2079 reporter plasmid (data not shown). In addition, TSA failed to stabilize the mRNA products of these vectors, suggesting that a HDI responsive element may exist at the extreme 3'-end of p21WAF1 mRNA (data not shown). To address this possibility, vectors were produced that harbored deletions in the extreme 3' region of the p21WAF1 3'UTR: -210WAF1 + p21WAF1 3'UTR 591-2064, -210WAF1 + p21WAF1 3'UTR 591-2031, -210WAF1 + p21WAF1 3'UTR 591-2000, and -210WAF1 + p21WAF1 3'UTR 591-1979 (Figure 4.26.). Remarkably, transient transfections demonstrated that deletions within as little as 79 base pairs from the 3' end of the p21WAF1 mRNA 3' UTR had a marked effect on basal mRNA stability (Figure 4.27.A). Nucleotides 2000-2079 appeared to possess significant destabilizing ability. Moreover, TSA (1.0  $\mu$ M) and NaB (5.0 mM) failed to enhance CAT levels of vectors with extreme 3' deletions to the same degree as the full length p21WAF1 3'UTR (FL), suggesting that a second *cis*-acting HDI responsive sequence may reside between nucleotides 2000-2079 (Figure 4.27.B, C). In spite of these observations, a -210WAF1-CAT + p21WAF1 3'UTR 1468-2079 vector was unable to reinstate HDI mediated mRNA stabilization of p21WAF1. Therefore, in accordance with our previous results, at least two *cis*-acting elements, located between 932-1272 and 2000-2079 of the p21WAF1 3' UTR are important sequence determinants of basal p21WAF1 mRNA stability and HDI induced p21WAF1 mRNA stability.

#### **4.3.4.4. p21WAF1 3'Untranslated Region Mutants**

A sequence alignment of the human p21WAF1 3' UTR 2001-2079 performed with corresponding rat and mouse p21WAF1 3' UTR sequences demonstrated a high degree of sequence conservation among these three species, further lending support to

-210WAF1-CAT + p21WAF1 3'UTR 591-2079 (FL)

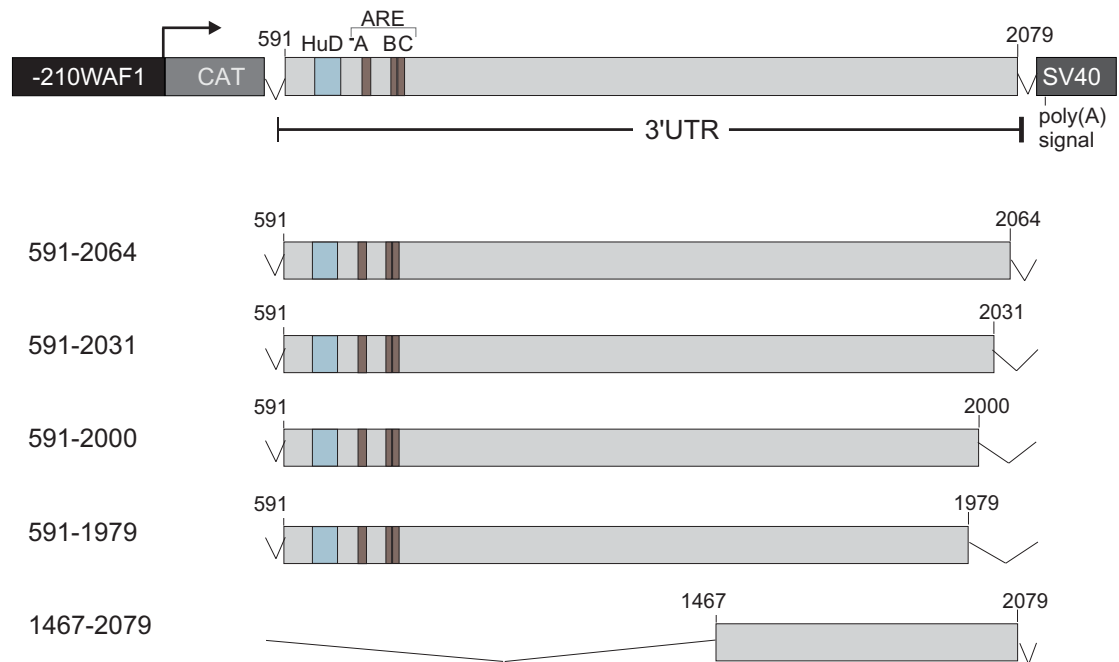


Figure 4.26. The -210WAF1-CAT + p21WAF1 3' UTR extreme 3' deletions. The p21WAF1 3' UTR sequence deletions were cloned into the -210WAF1-CAT vector downstream of the CAT coding region and upstream of the late SV40 poly(A) region and signal, as illustrated for the 591-2079 (FL) vector.

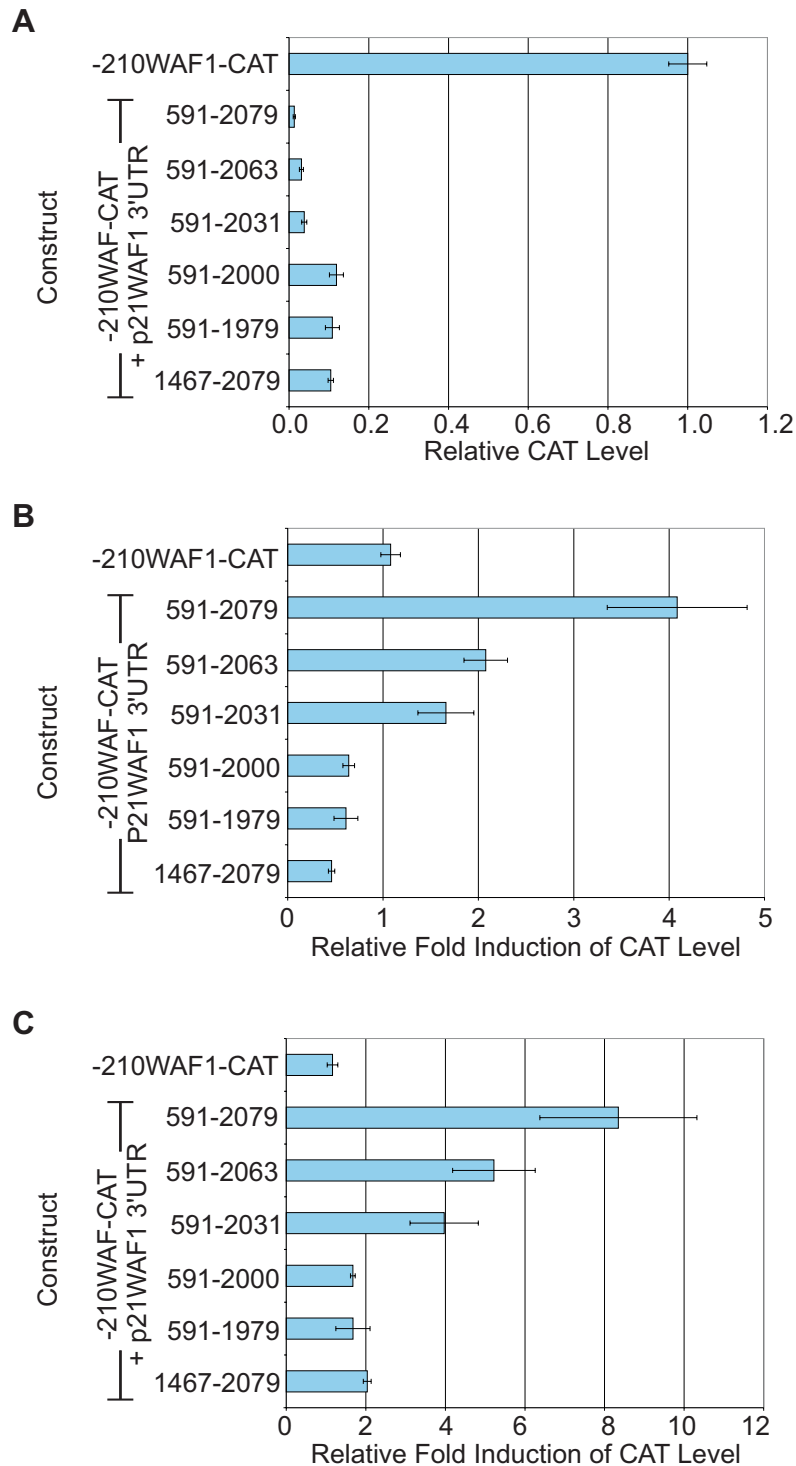


Figure 4.27. 3' Deletions of the p21WAF1 3' UTR suggest the presence of a second *cis*-acting element. (A) Transient transfection of HepG2 cells with empty -210WAF1-CAT or -210WAF1 + p21WAF1 3'UTR 3' deletions were performed and the basal relative fold induction of CAT levels determined. HepG2 transfected cells treated with TSA (1.0  $\mu$ M) (B) or NaB (5.0 mM) (C) are shown as fold induction relative to untreated self. Transfections are represented as the average of a minimum of two experiments carried out in duplicate and include standard deviations shown as error bars.

the notion that this region of the 3' UTR may be critical for mRNA stabilization (Figure 4.28.A). Therefore, to precisely pinpoint the identity of the HDI responsive element between nucleotide positions 2001-2079 of the 3' UTR, sixteen p21WAF1 mutant sequences were generated within this region. The wt base pairs 2001-2077 were replaced, in the -210WAF1 + p21WAF1 3'UTR (FL) vector, with p21WAF1 3' UTR sequence mutated at five consecutive base pairs per mutant plasmid, where pyrimidines were replaced with pyrimidines (C to T, or T to C) and purines with purines (A to G, G to A) (Figure 4.28.B, C). For example, p21WAF1 3'UTR mutant #1 (mut#1) had mutated sequence from 2001-2005, but 2006-2077 nucleotide pairs corresponded to wt p21WAF1 3' UTR sequence.

Transient transfections were initially performed with mut #1 through mut #8 reporter vectors, and compared to the empty -210WAF1-CAT vector in HepG2 cells. Furthermore, HepG2 cells were also transiently transfected with -210WAF1-CAT + p21WAF1 3'UTR (FL) and -210WAF1-CAT + p21WAF1 3'UTR 591-2000 to serve as benchmarks for depleted and elevated p21WAF1 basal mRNA stability, respectively. Each mutant exhibited low basal CAT levels that most closely resembled the CAT levels produced by the -210WAF1-CAT + p21WAF1 3'UTR 591-2079 (FL) plasmid (Figure 4.29.A). In addition, treatment with either TSA (1.0  $\mu$ M) or NaB (5.0 mM), did not compromise the mRNA stabilization of any of these mutants when judged against the -210WAF-CAT and -210WAF1 + p21WAF1 3'UTR 591-2000 vectors (Figure 4.29.B, C). Therefore, the nucleotide sequence between 2001-2040 of the p21WAF1 3' UTR does not appear to contain either a mRNA instability determinant, nor an HDI responsive element.

When -210WAF1-CAT + p21WAF1 3'UTR mut#9 through mut#16 were investigated in HepG2 transient transfections followed by CAT assays, however, a slight increase in the basal CAT levels of mut#15 and mut#16 plasmids was detected (Figure 4.30.A). Furthermore, following treatment with TSA (1.0  $\mu$ M) or NaB (5.0 mM), a reduction in mRNA stability was observed in HepG2 cells transiently transfected with the -210WAF1-CAT + p21WAF1 3'UTR mut#15, when compared to the -210WAF1 + p21WAF1 3'UTR 591-2000 vector (Figure 4.30.B, C). mRNA stability of mut#15 was enhanced approximately 2-fold and 4-fold by TSA and NaB,

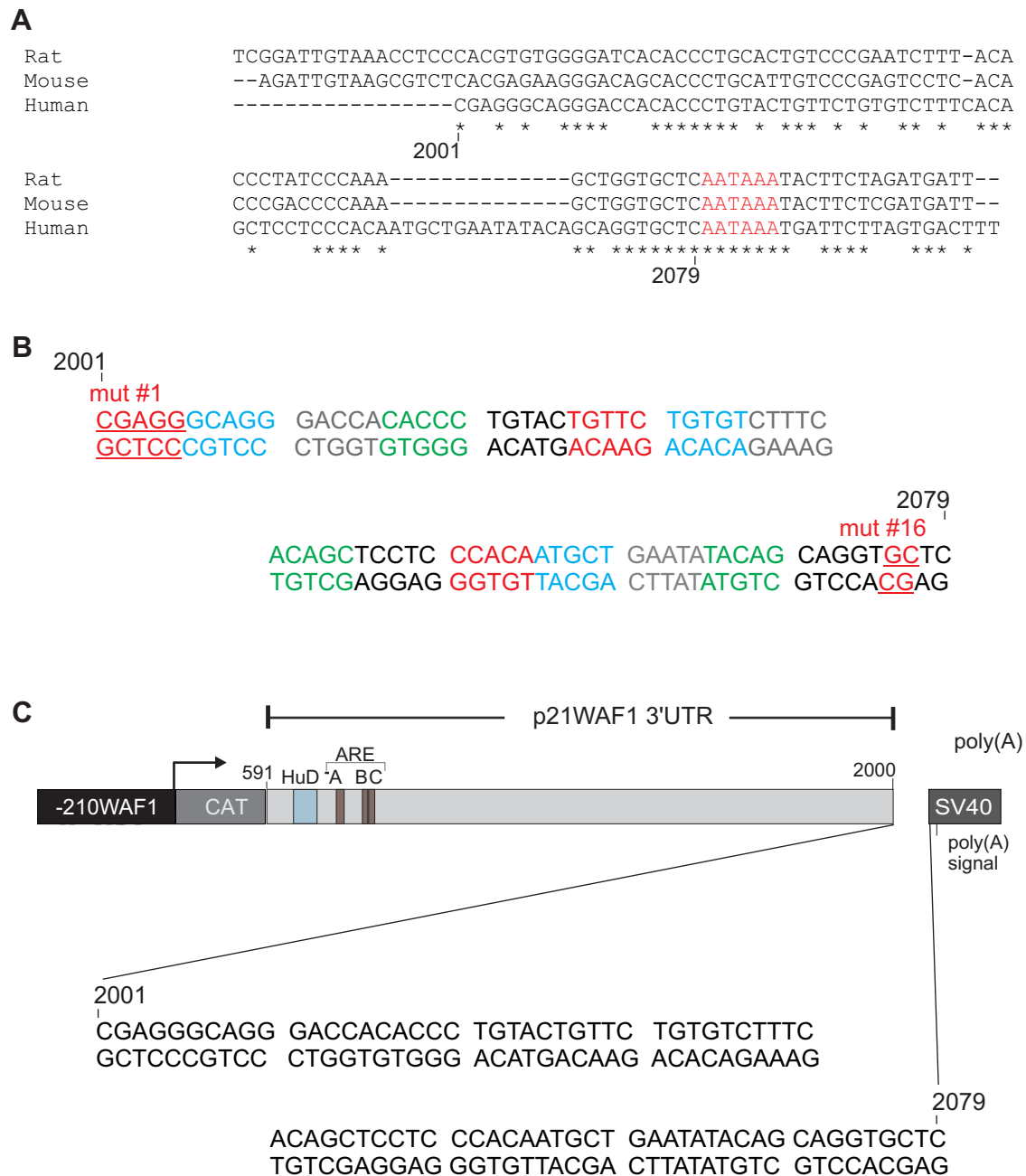


Figure 4.28. High sequence conservation exists within the extreme 3' region of the p21WAF1 3' UTR. (A) Sequence alignment results of rat (U24174), mouse (U24173) and human (U03106) p21WAF1 3' UTRs 85 base pairs upstream of their poly(A) hexamer signals. The hexamer signals are shown in red. (B) p21WAF1 3' UTR mutant design, where pyrimidines were replaced with pyrimidines and purines with purines. Starting at 2001 of the p21WAF1 3'UTR with mutant #1 (mut#1), five bases were mutated per p21WAF1 3'UTR mutant. The sequence to be altered for mut#1 is underlined and the other subsequent 5 bp mutations are denoted by color, leading up to mut#16 where only 2 bp are modified (underlined). (C) A representation of wt p21WAF1 3'UTR sequence 2001-2079 ligated into a -210WAF1-CAT + p21WAF1 3'UTR linearized plasmid lacking the 2001-2079 region of the p21WAF1 3'UTR.

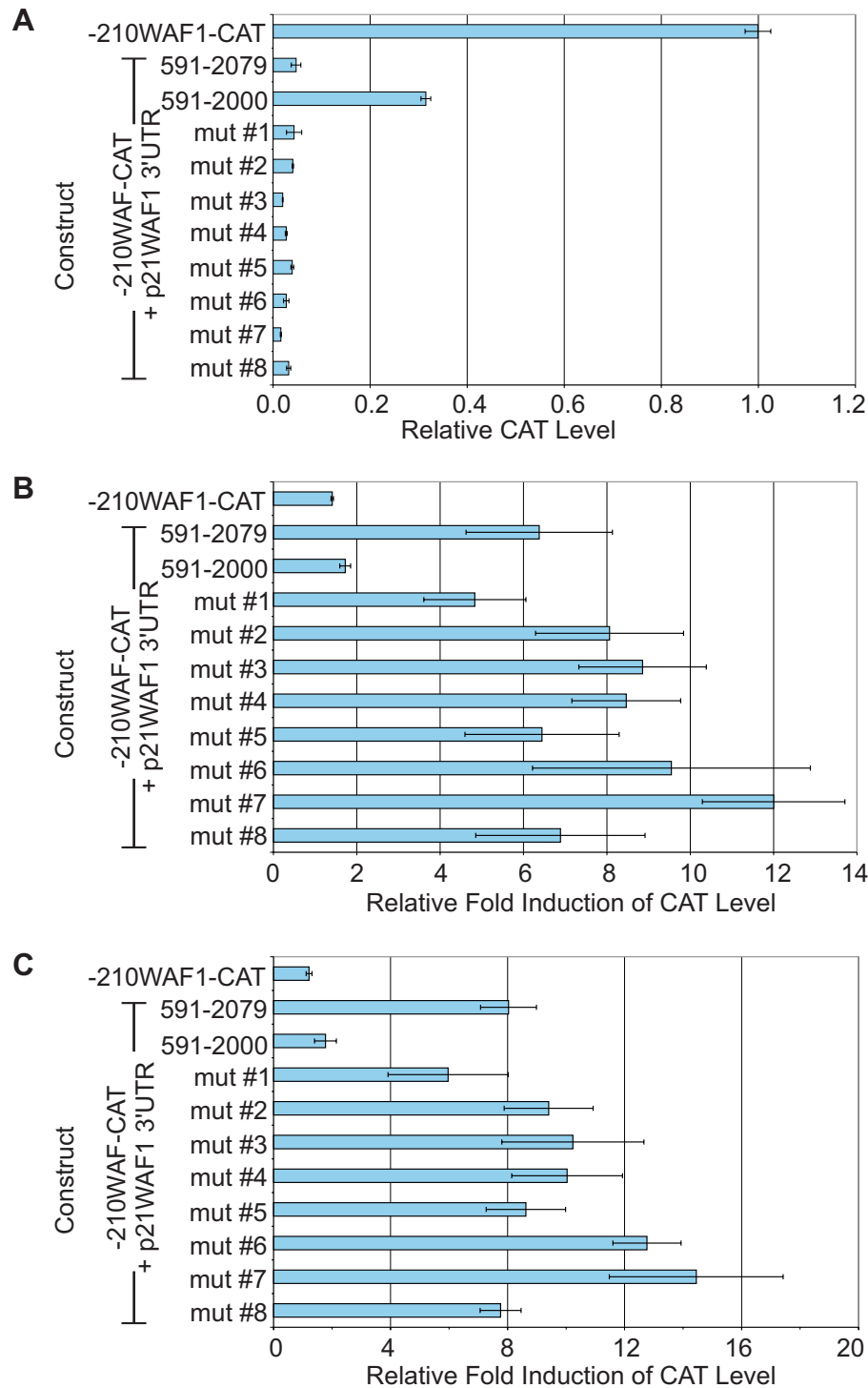


Figure 4.29. p21WAF1 3' UTR mutants #1 through # 8 are still capable of enhancing mRNA stability. Transient transfections were performed in HepG2 cells with empty -210WAF1-CAT, -210WAF1-CAT + p21WAF1 3'UTR 591-2079, 591-2000, or mutants (mut) #1 through #8. The basal relative fold induction of CAT levels were determined (A), as well as the fold induction relative to untreated self following TSA (1.0  $\mu$ M) (B) or NaB (5.0 mM) (C) treatment of the transfected cells. These experiments are an average of at least two independent experiments performed in duplicate and include error bars for standard deviations.

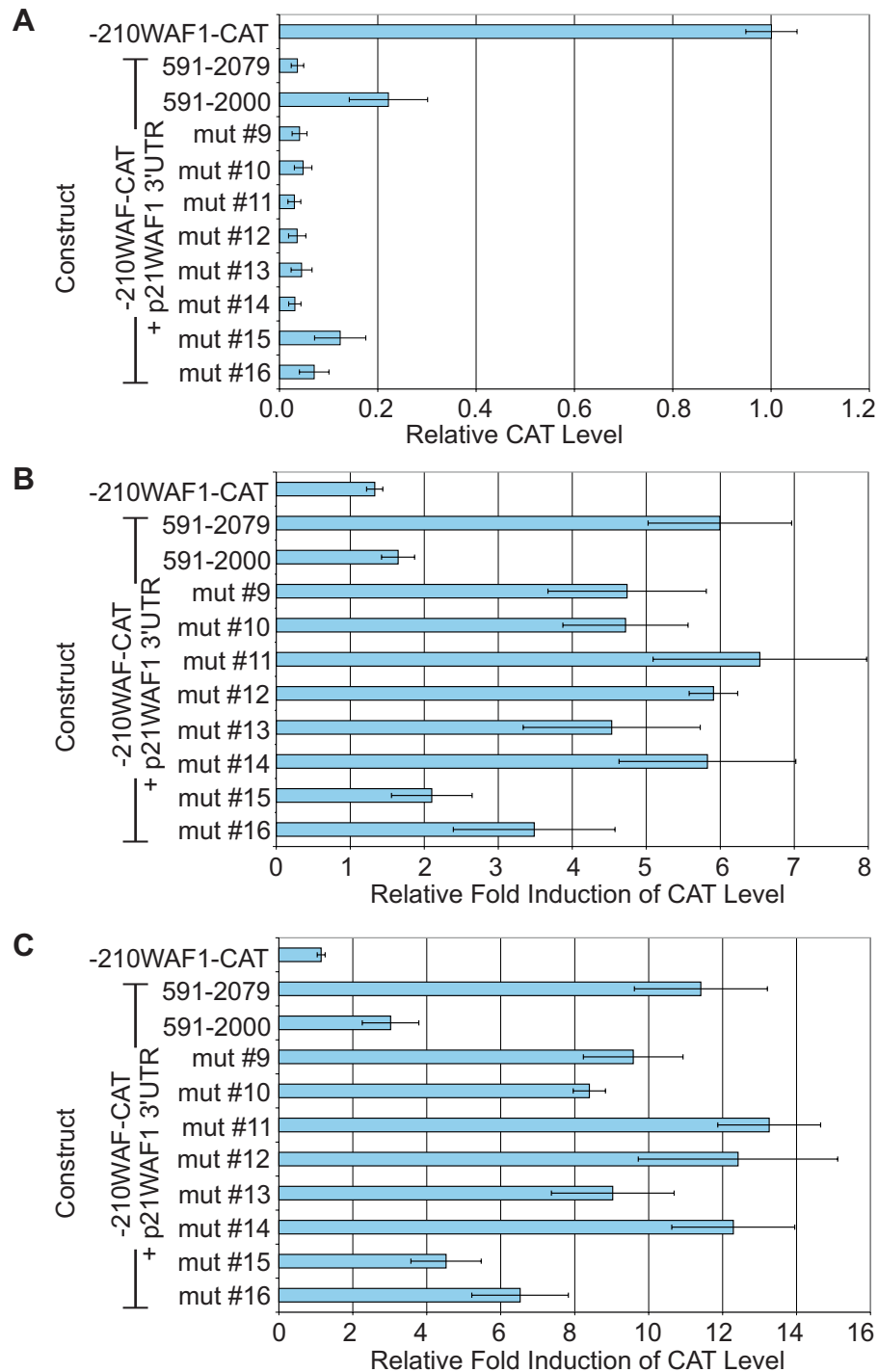


Figure 4.30. The p21WAF1 3' UTR mutants #15 and #16 have compromised mRNA stabilization ability in the presence of histone deacetylase inhibitors. HepG2 transient transfections were carried out with empty -210WAF1-CAT, -210WAF1-CAT + p21WAF1 3'UTR 591-2079, 591-2000, or mutants (mut) #9 through #16. The basal relative fold induction of CAT levels were determined (A), as well as the fold induction relative to untreated self following TSA (1.0  $\mu$ M) (B) or NaB (5.0 mM) (C) treatment of the transfected cells. The average of a minimum of two transfection experiments, performed in duplicate, are displayed. Standard deviations are shown as error bars.

respectively, compared to a 1.5-fold and 3-fold increase following TSA and NaB exposure to -210WAF1 + p21 3'UTR 591- 2000 transfections. A decrease in mRNA stability was also evident in mut#16 transfections, but not to the same degree as in mut#15 transfections. Cumulatively, these data suggest that the region between nucleotides 2071-2077 of the p21WAF1 3' UTR, contain an instability determinant and a responsive element that may be responsible for HDI mediated stabilization of the p21WAF1 mRNA in HepG2 cells.

The poly(A) signal hexamer sequence of the p21WAF1 mRNA resides between nucleotides 2080-2085, which falls adjacent to the 2071-2077 *cis*-acting element implicated in p21WAF1 mRNA stability. Given that all of the previous -210WAF1-CAT + p21WAF1 3'UTR plasmids were missing the endogenous p21WAF1 poly(A) signal hexamer motif, but utilized the CAT vector SV40 late poly(A) region and signal, it was vital to exclude the possibility that the changes in p21WAF1 mRNA stability observed in mut#15 and mut#16 HepG2 transfections were the result of the absence of the endogenous p21WAF1 poly(A) signal hexamer. Three vectors containing the original p21WAF1 poly(A) AATAAA hexamer, rather than the downstream SV40 late poly(A) signal, were generated: -210WAF1-CAT + p21WAF1 3'UTR wt poly(A) (Figure 4.31.), -210WAF1-CAT + p21WAF1 3'UTR mut#15 poly(A), and -210WAF1-CAT + p21WAF1 3'UTR mut #15/#16 poly(A). In contrast to the previous experiments, mut #15/#16 poly(A) contained nine mutated base pairs, from 2071-2079. Transient transfections were carried out with these vectors in HepG2 cells, exactly as in earlier experiments. The basal CAT levels and relative fold induction following TSA (1.0  $\mu$ M) and NaB (5.0 mM) exposure were compared to the original plasmids lacking the p21WAF1 poly(A) signal hexamer sequence. Indeed the relative CAT levels of untreated wt poly(A) and mut#15 poly(A) vectors matched those of the -210WAF1-CAT + p21WAF1 3'UTR 591-2079 (FL) and -210WAF1-CAT + p21WAF1 3'UTR mut#15 first series plasmids, respectively (Figure 4.32.A). In addition, wt poly(A) CAT levels were induced by TSA (B) and NaB (C), although to a lesser extent than the -210WAF1-CAT + p21WAF1 3'UTR 591-2079 (FL) vector (Figure 4.32.). Also, CAT levels of mut#15 poly(A) and -210WAF1-CAT + p21WAF1 3'UTR mut#15 reporter plasmids were similarly suppressed by HDIs, especially TSA. Interestingly, mut



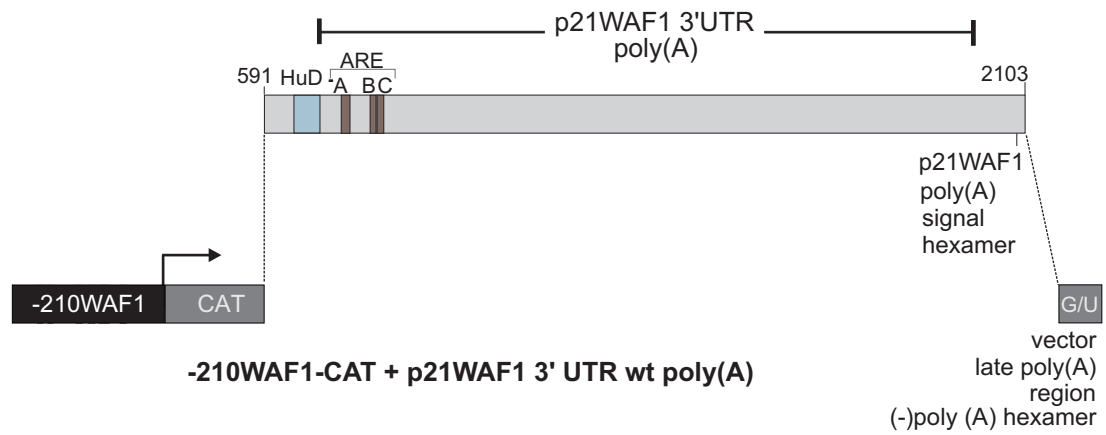


Figure 4.31. A diagram of the **-210WAF1-CAT + p21WAF1 3'UTR wt + poly(A)** hexamer vector. An enlarged fragment of the p21WAF1 3' UTR was cloned into the -210WAF1-CAT plasmid downstream of the CAT coding region and upstream of the late SV40 Poly (A) region lacking a poly(A) signal hexamer, but still containing the GU-rich element.

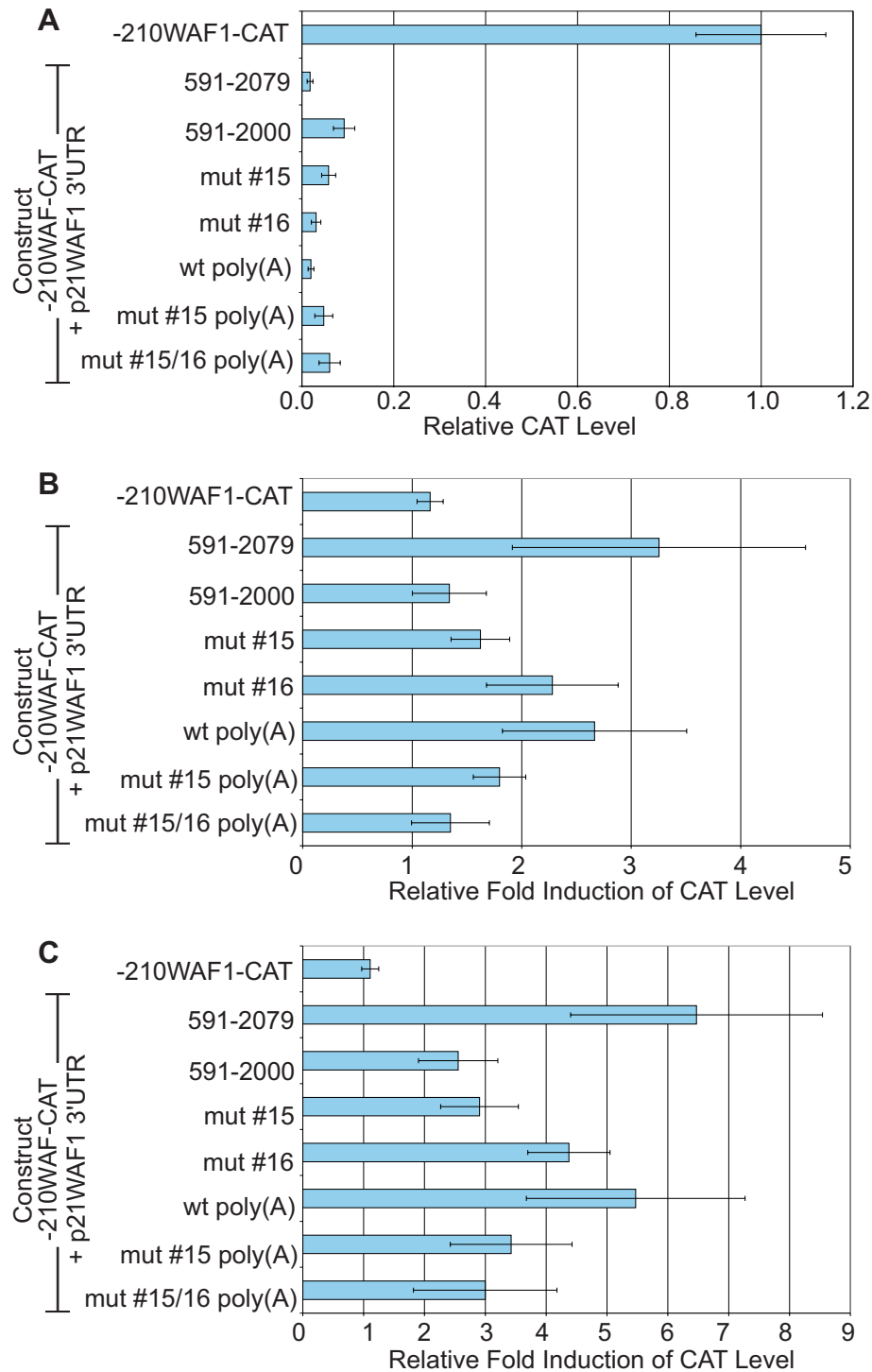


Figure 4.32. Nucleotides 2071-2079 of the p21WAF1 3' UTR contain an instability determinant and a histone deacetylase inhibitor responsive element. HepG2 transient transfections were performed and the relative fold basal induction of CAT levels were determined (A), in addition to the fold induction relative to untreated self upon TSA (1.0  $\mu$ M) (B) or NaB (5.0 mM) (C) exposure to transfected cells. Constructs containing the p21WAF1 poly(A) hexamer are denoted by poly(A). The average of three transfection experiments, performed in duplicate are displayed with error bars for standard deviations.

#15/#16 poly(A) CAT levels were induced approximately 1.5-fold following TSA treatment, analogous to what was observed with the -210WAF1-CAT and -210WAF1-CAT + p21WAF1 3'UTR 591-2000 control vectors (Figure 4.32.B, C). NaB upregulated both -210WAF1-CAT + p21WAF1 3'UTR 591-2000 and mut#15/#16 poly(A) roughly 3-fold, while the activity of the parental -210WAF1-CAT reporter plasmid was not induced by NaB. An ongoing observation throughout the course of these experiments has been that NaB produces higher CAT gene expression levels than TSA in HepG2 cells, suggesting that NaB may elicit other effects to influence p21WAF1 mRNA decay beyond its activity as a HDI. Taken as a whole, these observations indicate that inclusion of the p21WAF1 poly(A) signal hexamer does not influence p21WAF1 mRNA stability levels in the absence or presence of HDIs. Furthermore, the region directly adjacent to the p21WAF1 poly(A) hexamer, between 2071 and 2079 of the p21WAF1 3' UTR, contains a stability determinant that is responsive to HDIs in HepG2 cells.

Cumulatively, this work strongly suggests that there are multiple *cis*-acting elements in the p21WAF1 3' UTR that influence mRNA decay; one between 932-1272 and another encompassing 2071-2077 have been characterized. mRNA stability is known to be regulated by *trans*-acting factors that bind *cis*-acting stability determinants within the 3' UTR of mRNAs. In an effort to identify differences in *trans*-RNA binding factors across the length of the p21WAF1 mRNA 3' UTR, RMSAs were performed. HepG2 cytoplasmic (A) and nuclear (B) extracts isolated in the absence or presence of TSA (1.0  $\mu$ M) were incubated with three RNA probes equivalent to sections of the p21WAF1 3' UTR: ribonucleotides 586-840, 840-1481 and 1476-2079 (Figure 4.33.). *Trans*-proteins were shown to associate with the p21WAF1 3' UTR; however, this method failed to consistently recognize changes in *trans*-factors interacting with the p21WAF1 3' UTR following HDI treatment. Therefore, in combination with earlier results, mRNA secondary structure may play an important role in this HDI mediated mRNA stabilization effect, and likely requires the entire context of nucleotides 932-2079 of the p21WAF1 3' UTR.

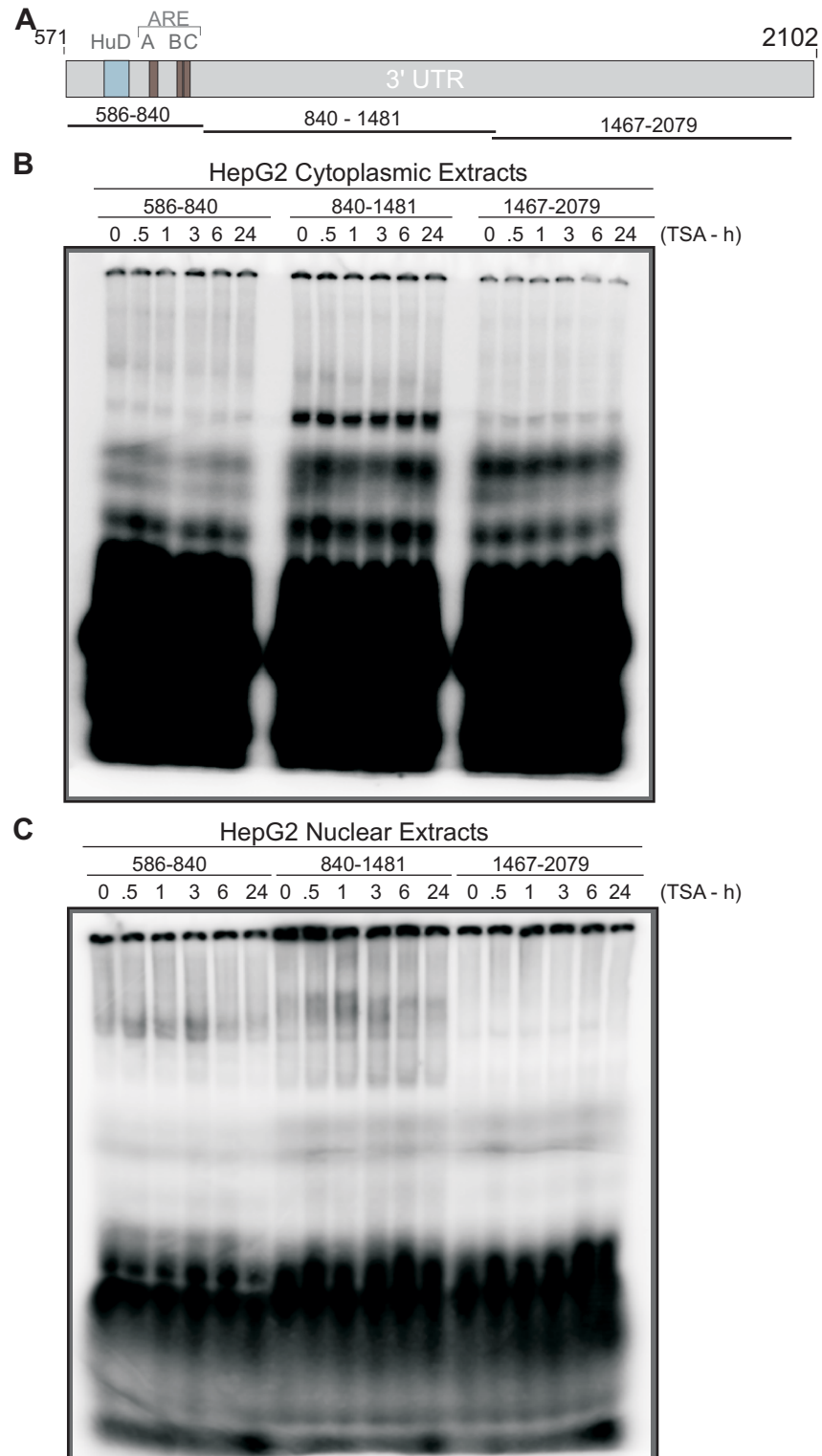


Figure 4.33. A change in *trans*-binding factors to the 3' UTR of p21WAF1 mRNA does not occur following histone deacetylase inhibitor treatment. (A) Three RNA probes corresponding to the p21WAF1 mRNA 3' UTR were generated for RMSAs; 586-840, 840-1481, and 1467-2079. Cytoplasmic (B) and (C) nuclear extracts were isolated from either untreated HepG2 cells or those treated with TSA (1.0  $\mu$ M), incubated with  $^{32}$ P-labelled p21WAF1 3'UTR RNA probes and resolved by RMSA.

#### 4.3.5. SUMMARY

HDIs have been well documented to directly enhance p21WAF1 gene expression at the transcriptional level (Nakano *et al.*, 1997, Sowa *et al.*, 1997). However, for the first time, new protein synthesis has been elucidated as a requirement for HDI mediated upregulation of p21WAF1 mRNA levels in HepG2 cells. In addition, it was shown that HDIs are incapable of activating the p21WAF1 promoter while p21WAF1 mRNA levels are stabilized by both NaB and TSA in HepG2 cells. These studies represent the first example of HDI mediated mRNA stabilization (Hirsch and Bonham, 2004). The 3' UTR of the p21WAF1 mRNA has been extensively implicated in mRNA stabilization (el-Deiry *et al.*, 1993, Li *et al.*, 1996, Joseph *et al.*, 1998, Liu *et al.*, 2000, Wang *et al.*, 2000, Giles *et al.*, 2003, Lal *et al.*, 2004 and Yang *et al.*, 2004). By linking the p21WAF1 3' UTR to the downstream end of a p21WAF1-CAT reporter gene, the p21WAF1 mRNA 3' UTR was demonstrated to be an important contributor to mRNA stability in the absence and presence of HDIs. Many 3' UTR deletion and mutant plasmids were generated, which identified two previously unreported *cis*-acting stability elements between 932-1272 and 2071-2079 in the p21WAF1 3'UTR that mediated basal and HDI effects on p21WAF1 mRNA stability (Table 4.1.)

Reporter Vector: -210WAF1 + p21WAF1 3'UTR	Basal CAT Activity (%)	TSA - Fold Induction CAT Activity	NaB - Fold Induction CAT Activity
-210WAF1-CAT	↑↑	↓	↓
591-2079 (FL)	↓	↑↑	↑↑
712-2079	↓	↑↑	↑↑
788-2079	↓	↑↑	↑↑
840-2079	↓	↑	↑↑
591-840	↑↑	↓	↓
932-2079	↓	↑↑	↑↑
1045-2079	↓	↓	↑↑
1173-2079	↑	↓	↑↑
1272-2079	↑	↓	↑↑
840-2079	↑	↓	↑
591-2063	↑	↓	↑↑
591-2031	↑	↓	↑
591-2000	↑	↓	↓
591-1979	↑	↓	↓
1467-2079	↑	↓	↓
mut#1	↓	↑↑	↑↑
mut#2	↓	↑↑	↑↑
mut#3	↓	↑↑	↑↑
mut#4	↓	↑↑	↑↑
mut#5	↓	↑↑	↑↑
mut#6	↓	↑↑	↑↑
mut#7	↓	↑↑	↑↑
mut#8	↓	↑↑	↑↑
mut#9	↓	↑↑	↑↑
mut#10	↓	↑↑	↑↑
mut#11	↓	↑↑	↑↑
mut#12	↓	↑↑	↑↑
mut#13	↓	↑↑	↑↑
mut#14	↓	↑↑	↑↑
mut#15	↑	↓	↑
mut#16	↑	↑	↑↑
wt poly(A)	↓	↑	↑↑
mut#15 poly(A)	↑	↓	↑
mut#15/#16 poly(A)	↑	↓	↑

Table 4.1. A summary table comparing relative basal CAT activities and CAT fold induction following HDI exposure in HepG2 transfected cells with the -210WAF1-CAT + p21WAF1 3' UTR deletion and mutation plasmids. Basal CAT activity; ↑↑ ≥ 50%, ↑ ≥ 5%, ↓ < 5%. Fold induction following HDI treatment; ↑↑ > 4-fold, ↑ > 2-fold, ↓ ≤ 2-fold.

## **5. GENERAL DISCUSSION**

### **5.1. Histone Deacetylase Inhibitors Function as Anti-neoplastic Agents**

A considerable amount of effort has been invested in clinical trials of the anti-tumor HDI compounds. Due to their ability to selectively induce cell cycle arrest, differentiation and/or apoptosis of tumor cells *in vitro* and *in vivo*, while showing little toxicity to normal cells, these compounds make attractive chemo-therapeutic agents (Marks *et al.*, 2001; Vigushin and Coombes, 2002 and Arts *et al.*, 2003). Three HDIs have currently been approved for clinical use: butyrate, valproic acid and the hydroxamic acid SAHA. Interestingly, both butyrate and valproic acid were originally approved by the FDA for the treatment of urea cycle disorders and epilepsy, respectively (Rosato and Grant, 2004). However, in the past year SAHA became the first HDI approved for clinical use in the treatment of a human malignancy, cutaneous T-cell lymphoma (CTCL) (Garber, 2007 and Marks and Breslow, 2007). In addition, an application for FDA approval of the HDI depsipeptide, as a therapeutic agent for CTCL and peripheral T-cell lymphoma (PTCL), is anticipated within the year (Garber, 2007). Currently, at least eight HDIs are the focus of multiple Phase I and Phase II clinical trials, either as primary or combinatorial treatments for various hematological and solid tumors. Given the promising collection of clinical trial data and the recent clinical recognition of SAHA, HDIs are rapidly proving themselves as worthy anti-cancer agents.

Although the study of HDIs appears to be gaining momentum in the clinical setting, the comprehension of how HDIs direct tumor cells to undergo growth arrest, as well as enhance cellular differentiation and apoptosis is lagging behind considerably. Various signaling pathways involved in eliciting HDI mediated effects have become more clearly understood, such as HDI activation of the mitochondrial apoptotic pathway

and stimulation of cell cycle arrest, but the molecular mechanisms which underlie such signaling cascades and achieve these anti-tumor effects are poorly understood (Bolden *et al.*, 2006). The cyclin dependent kinase inhibitor gene, p21WAF1, is both transcriptionally activated by HDIs and an absolute requirement for HDI induced cell cycle arrest in human tumor cells (Nakano *et al.*, 1997 and Archer *et al.*, 1998). Given these observations, p21WAF1 has been typecast as the model gene studied to understand the mechanisms of HDI action.

## **5.2. The Mechanisms of Histone Deacetylase Inhibitor Action**

HDIs have been proposed to carry out their chemotherapeutic actions by altering the expression levels of genes associated with the cell cycle, differentiation and apoptosis. As inhibitors of the histone modifying enzymes, HDACs, HDIs are primarily thought to regulate the gene expression pattern of tumor cells by interfering with the overall balance of histone acetylation levels. Direct inhibition of HDAC activity shifts the histone acetylation status towards hyperacetylation, which is anticipated to activate transcription and enhance gene expression by relaxing the chromatin structure and allowing PIC and transcription factor access to the DNA promoter. This theory not only fits well with the observation that a number of HATs, such as PCAF and p300, behave as co-activators while HDACs function as co-repressors, but a global elevation in H4 and H3 acetylation levels following HDI exposure correlates with growth arrest at the G1 and G2/M phases of the cell cycle in tumor cells (Sowa *et al.*, 1997; Saito *et al.*, 1999; Han *et al.*, 2000 and Han *et al.*, 2001). Furthermore, examination of the p21WAF1 gene also initially corroborated this theory when it was reported that HDIs directly enhanced the overall H3 and H4 acetylation levels in chromatin associated with the p21WAF1 promoter and downstream gene, and that the p21WAF1 promoter was activated in a p53 independent manner that required the presence of at least one of the Sp family binding sites (Nakano *et al.*, 1997; Sowa *et al.*, 1997; Huang *et al.*, 2000; Richon *et al.*, 2000 and Kim *et al.*, 2001). In addition, the HAT p300 was identified as an activator of the p21WAF1 promoter, while HDAC1, HDAC2 and HDAC3 were implicated as repressors (Xiao *et al.*, 2000; Lagger *et al.*, 2003; Gui *et al.*, 2004; Huang *et al.*, 2005 and Wilson *et al.*, 2006). Taken together, these observations strongly



suggest that the anti-tumor effects carried out by HDIs are the result of increased histone acetylation and transcriptional upregulation of genes.

However, when microarray profiling was performed to identify genes affected by HDIs, only a small and selective subset of genes (2-20%) were found to be altered. Moreover, genes were both activated and suppressed by HDIs in equivalent proportions (Van Lint *et al.*, 1996; Mariadason *et al.*, 2000 and Peart *et al.*, 2005). If HDIs purely elicited their anti-tumor effects by histone hyperacetylation and subsequent gene activation, a far greater percentage of expression profiles would be expected to be affected by exposure to HDIs. Since these observations, the p21WAF1 model gene, used to study the molecular mechanism of HDIs, has also undergone some revision. An upstream signaling pathway involving PI 3-kinase and PKC $\epsilon$  isoform has now been implicated in HDI mediated upregulation of the p21WAF1 promoter through the Sp1-3 site (Han *et al.*, 2001 and Kim *et al.*, 2003). Furthermore, H3 acetylation alone was determined to be an insufficient means of stimulating the p21WAF1 promoter in the absence of PKC activity (Kim *et al.*, 2003). In addition, histone acetylation has been proposed to be a secondary effect of HDIs, as the alteration of H3 and H4 acetylation levels and chromatin rearrangement of the mouse mammary tumor virus (MMTV) promoter does not correlate with the massive transcriptional defect imposed by TSA (Mulholland *et al.*, 2004). Also, PKC $\epsilon$  and PKC $\delta$  isoforms have recently been reported as essential proteins in HDI mediated downregulation of gelsolin gene expression and upregulation of cyclin D3 transcription, respectively (Eun *et al.*, 2007 and Kim *et al.*, 2007). Therefore, it is apparent that the underlying mechanisms of HDI action need to be revisited to account for HDI mediated gene repression and upregulation in the absence of histone acetylation. This thesis has focused specifically on elucidating the effects of HDIs on the expression of the c-Src proto-oncogene, the SFK members and p21WAF1, outside of the context of gene activation mediated by histone hyperacetylation and chromatin rearrangement. Simply, the data within demonstrates that HDIs repress the mRNA and protein expression levels of c-Src and the SFK members in HCCLs, and can enhance p21WAF1 mRNA levels at the post-transcriptional level in HepG2 cells.

### **5.3. Histone Deacetylase Inhibitor Repression of c-Src and the c-Src Family Kinase Members**

#### **5.3.1. c-Src Gene Expression is Suppressed by Histone Deacetylase Inhibitors**

As reported in this thesis, HDIs were determined to be proficient inhibitors of c-Src gene mRNA and protein expression levels in a variety of human tumor cell lines, including colon, breast and hepatocellular carcinoma cells. This HDI mediated effect on c-Src expression, which has been observed in all tumor cell lines examined to date, occurred independently of new protein synthesis and directly inhibited SRC transcription. Surprisingly, HDIs independently and similarly repressed expression from both the highly dissimilar SRC1 $\alpha$  and SRC1A promoters by approximately 80-95%. Both TSA and butyrate, a noncompetitive HDI, comparably suppressed these promoters and activated the p21WAF1 promoter, indicating that inhibition of HDAC activity was responsible for the downregulation of c-Src expression. Gene expression analysis drew attention to the fact that a tight correlation existed between HDI increases in p21WAF1 expression and decreases in c-Src expression levels. However, p21WAF1 was not responsible for HDI mediated inactivation of the SRC1 $\alpha$  or SRC1A promoter. In fact, p21WAF1 activated both SRC promoters in HepG2 cells, which is a particularly interesting observation given that p21WAF1 has previously been reported to behave as a transcriptional cofactor and transcriptional regulator of genes including c-myc, cdc25 and IGF-I (Perkins, 2002; Devgan *et al.*, 2006 and Vigneron *et al.*, 2006).

#### **5.3.2. c-Src Family Kinase Member Expression is Downregulated by Histone Deacetylase Inhibitors**

In addition to c-Src, expression levels of the ubiquitously expressed SFK members, Yes and Fyn, and the more tissue specific Lck and Lyn were also shown to be repressed by HDIs in various HCCLs. Both butyrate and TSA suppressed the mRNA levels of these SFKs after 24 h of HDI exposure. As a rule, Src, Fyn and Lck expression levels were downregulated by butyrate in a dose dependent manner, while Yes and Lyn often displayed a transient increase in mRNA levels approximately 6 h after butyrate exposure. Yes and Lyn were consistently also repressed by 24 h, however, TSA time course experiments verified that the HDI activity of butyrate was responsible for both the transient upregulation and ultimate decrease of SFK mRNA expression levels.

Furthermore, as observed with c-Src, butyrate directly downregulated Lck and Lyn mRNA levels in HCCLs, independently of protein neosynthesis. HDI mediated suppression of Yes mRNA expression could not conclusively be reported to occur in the absence of protein synthesis, however, remarkably, the LCK type I promoter utilized in colon cancer tissues, LYN and YES promoters were all downregulated by butyrate and TSA in transient transfections. HDI mediated repression of the SFK member promoters was observed as early as 6 h following HDI treatment and was maintained for the full 24 h period. Although the LYN promoter was consistently inhibited by HDIs, butyrate mediated suppression of this promoter was not as striking after 12 and 24 h when compared to transfected cells treated with butyrate for only 6 h. It is important to note that the transcriptional activity of the YES and LYN promoters were not transiently stimulated by butyrate or TSA. Therefore, the temporary induction observed in Yes and Lyn mRNA levels may be the result of two HDI mediated processes. Firstly, HDIs may increase histone hyperacetylation of the chromatin template to facilitate chromatin rearrangement and transcriptional upregulation. However, this increase in transcriptional activity may not be detected in transient transfections given that complex chromatin structure is not readily formed in this experimental system. Alternatively, HDIs may enhance mRNA stabilization of these SFK members, since three separate reports have now indicated that HDIs harbor the ability to post-transcriptionally regulate gene expression (Hirsch and Bonham, 2004; Xiong *et al.*, 2005 and Januchowski *et al.*, 2007).

### **5.3.3. The Mechanism of Histone Deacetylase Inhibitor Directed Inhibition of c-Src and the c-Src Family Kinase Members**

The molecular mechanism of HDI mediated suppression of c-Src and SFK member expression levels has been most thoroughly investigated using SRC as the model gene. First, proximal promoter mutants and deletions were made to the SRC1 $\alpha$  and SRC1A-CAT promoter vectors, to assess the importance of proximal promoter elements in this effect. Reports studying the response of the p21WAF1 promoter to HDIs have suggested that at least one of the Sp sites in the proximal promoter region is necessary to facilitate HDI activation (Nakano *et al.*, 1997; Sowa *et al.*, 1997 and Xiao *et al.*, 2000). Interestingly, the SRC1A, LYN and YES promoters also harbor at least

three Sp sites (Matsuzawa *et al.*, 1991; Uchiumi *et al.*, 1992; Bonham and Fujita, 1997 and Ritchie *et al.*, 2000). While mutations in the Sp binding sites GC1 and GA2 of the 0.38SRC1A-CAT reporter plasmid decreased promoter activity, HDIs always further repressed the SRC1A promoter (Kostyniuk *et al.*, 2002). Furthermore, extensive core and proximal promoter deletions were made to the 0.38SRC1A-CAT and -145SR1 $\alpha$ -CAT vectors in order to search for the presence of HDI responsive elements (Dehm *et al.*, 2004). However, HDIs still consistently downregulated SRC gene expression in every deletion plasmid examined (Dehm *et al.*, 2004). In addition to this, electrophoretic mobility shift assays demonstrated that HDI transcriptional inhibition of the SRC1 $\alpha$  and SRC1A promoters was not associated with either an increase or decrease in factors bound to their proximal promoter regions (Kostyniuk *et al.*, 2002). *In vivo*, ChIPs also verified that the presence of HDIs did not change the levels of Sp1 and Sp3 associated with the SRC1A promoter (Ellis and Bonham, unpublished results). Taken together, these results suggest that it is unlikely that a proximal promoter element exclusively mediates the effects of HDIs on c-Src expression levels.

#### **5.3.3.1. The Involvement of TAF1**

When the SRC gene and the SFK member gene promoters were examined more closely, two major similarities were noted: all of the SFK core promoters, SRC1A, SRC1 $\alpha$ , YES, LYN, and LCK type I, are TATA-less and all contain multiple transcription start sites (Matsuzawa *et al.*, 1991; Uchiumi *et al.*, 1992; Leung *et al.*, 1993 and Bonham and Fujita, 1997). Interestingly, a consensus sequence resembling an Inr element was identified and mapped to a major transcription start site in both the SRC1A and SRC1 $\alpha$  promoters (Dehm *et al.*, 2004). A characteristic of Inr element driven promoters is their ability to interact with TAF1-TAF2 heterodimers in order to nucleate assembly of the transcriptional PIC (Chalkley and Verrijzer, 1999). *In vitro*, both of the proposed SRC1A and SRC1 $\alpha$  Inr elements successfully bound to TAF1-TAF2 heterodimers (Dehm *et al.*, 2004). Furthermore, utilization of the matched cell lines BHK-21 and *tsBN462*, which contains a G690D temperature sensitive mutant of TAF1, demonstrated that the SRC1A promoter was TAF1 dependent in nature (Dehm *et al.*, 2004). In addition, at the restrictive temperature, HDIs were incapable of repressing the SRC1A promoter in *tsBN462* cells transiently transfected with 0.38SRC1A-CAT,

suggesting that TAF1 may also be responsible for HDI mediated repression of the SRC1A promoter (Dehm *et al.*, 2004). Similar experiments were performed with the SRC1 $\alpha$  promoter, however, the high restrictive temperature was shown to significantly lower the trans-activation of the promoter, making these results difficult to interpret (Dehm *et al.*, 2004). Yet, a chimeric promoter construct of the -210WAF1 proximal promoter fused to the SRC1 $\alpha$  Inr core promoter element conferred TAF1 dependence on the previously TAF1 independent p21WAF1 promoter (Dehm *et al.*, 2004). *In vitro* binding assays more specifically demonstrated that association of the G690D mutant TAF1-TAF2 heterodimer with the SRC1A and SRC1 $\alpha$  promoters diminished at the non-permissive temperature, while wt TAF1 binding remained consistent (Dehm *et al.*, 2004). Therefore, these results suggest that both the SRC1A and SRC1 $\alpha$  promoters are TAF1 dependent and hint that HDI mediated repression of SRC may involve TAF1.

Core promoter Inr elements have not yet been defined in the YES, LYN and LCK type I promoters, though potential consensus sequences suggest that Inr elements may be present in the core promoter regions of these SFKs. The identification of Inr elements in SFK member promoters is out of the scope of this thesis; however, given that TAF1 has been implicated in basal transcription of the SRC1A promoter and that SRC1A transcription is repressed by HDIs, TAF1 dependency on the YES, LYN and LCK type I gene promoters was assessed to establish the commonality of TAF1 function across the family of c-Src kinases. By performing transient transfection experiments in the parental BHK-21 and matched *tsBN462* cells with 0.38SRC1A, pYSCAT2, or -590LYN-CAT reporter plasmids, it was observed that both the SRC1A and YES promoters displayed a dependency on TAF1. The activity of each of these reporter plasmids was suppressed to some degree in parental BHK-21 cells at the restrictive temperature, although in every instance SFK promoter activity was even further inhibited in *tsBN462* cells at the same restrictive temperature which triggers both impaired TAF1 AT activity and SRC1A Inr element binding capacity. Overall, reductions of approximately 50% and 30% were observed in SRC1A and YES basal transcription, respectively, which accounts purely for compromised TAF1 AT activity and/or DNA binding ability. However, only a general 10% decrease in LYN promoter activity was observed. Although each of the SFK promoters examined displayed some

degree of temperature sensitivity, activity of the LYN promoter was inhibited over 80% at the restrictive temperature in BHK-21 cells, which may explain the minimal decrease observed in LYN transcription following TAF1 impairment. Furthermore, these data suggest that activity of the LYN promoter is temperature dependent, similar to the SRC1 $\alpha$  promoter (Dehm *et al.*, 2004). In addition, results from transient co-transfection experiments carried out with wt TAF1 indicated that SRC1A, YES and LYN promoter activity could be restored at the restrictive temperature in *tsBN462* cells, at least to levels previously observed for matched BHK-21 transfected cells exposed to the non-permissive temperature. Further co-transfections performed with various TAF1 deletions and mutants demonstrated that only those TAF1 expression plasmids that retained TAF1 AT activity were capable of rescuing SRC1A, YES and LYN promoter activity, while those TAF1 deletion vectors that lacked AT activity could not re-stimulate transcription. Therefore, each of these SFK member promoters is TAF1 dependent and requires TAF1 AT activity for basal transcription. To ensure that TAF1 AT activity was effectively separated from TAF1 Inr element binding, however, it must be demonstrated that each of the TAF1 deletions impaired in AT activity (TAF1  $\Delta$ Stu, TAF1  $\Delta$ MED) maintains their competence for binding the Inr element. A collaborator, Dr. Edith Wang, is currently performing binding experiments to verify that the TAF1 AT mutants remain able to bind the SRC1A Inr element at the restrictive temperature. Taken together, these data strongly suggest that TAF1 AT activity is necessary for basal SRC1A, YES and LYN transcription. While TAF1 is known to display relatively weak HAT activity towards histones H3 and H4, when compared to PCAF and p300, it is also capable of acetylating non-histone proteins such as the  $\beta$ -subunit of TFIIE (Imhof *et al.*, 1997). Therefore, TAF1 may negotiate SRC, YES and LYN transcription by modifying the chromatin structure associated with these genes and/or an unknown component of the transcriptional machinery.

Although relatively little work has been performed to uncover the potential molecular mechanisms of HDI mediated gene repression, one of the major proposals stipulates that HDIs may exert their anti-tumor effects by permitting HAT acetylation of non-histone proteins such as p53, GATA-1, E2F, YY1, Ku70 and STAT3 (Gu and Roeder, 1997; Boyes *et al.*, 1998; Kouzarides, 2000; Martinez-Balbas *et al.*, 2000; Yao

*et al.*, 2001; Cohen *et al.*, 2004; Glozak *et al.*, 2005; Yuan *et al.*, 2005 and Minucci and Pelicci, 2006). Acetylation of non-histone proteins has been reported to influence gene expression by affecting such factors as the DNA binding affinity of transcription factors, protein stabilization, protein dimerization and protein localization (Drummond *et al.*, 2005, Lin *et al.*, 2006 and Minucci and Pelicci, 2006). For example, acetylation of p53 by HDIs increases cell cycle arrest by stabilizing p53 protein levels and promoting activation of the p21WAF1 promoter (Zhao *et al.*, 2006). Additionally, such a mechanism of HDI mediated acetylation of non-histone proteins has been proposed to explain TSA repression of the MMTV promoter (Mulholland *et al.*, 2003).

Changes in the acetylation status of the histones H3 and H4 associated with the SRC1A promoter do not effectively correspond with HDI mediated SRC gene repression. This suggests that a shift in the balance between HAT and HDAC activity may be responsible for decreased SRC1A transcription through the acetylation of a non-histone protein linked to the SRC1A promoter (Ellis and Bonham, unpublished data). Previously, Dr. Scott Dehm had shown that TAF1 may be required for HDI mediated downregulation of the SRC1A promoter, which implies that TAF1 may be the HAT responsible for acetylating an as yet unrevealed non-histone substrate (Dehm *et al.*, 2004). However, given that the AT activity of TAF1 is imperative for basal transcription, it is counterintuitive that TAF1 may also be required to mediate SRC1A transcriptional repression in the presence of HDIs, particularly if the loss of TAF1 AT activity is responsible for preventing HDI downregulation of the SRC1A promoter. In accordance with this thought, a common role for TAF1 in HDI mediated inhibition of SFK member transcription does not appear to be prevalent, as three separate responses were generated by the SRC1A, YES and LYN SFK promoters upon TSA treatment in *tsBN462* cells exposed to the restrictive temperature. First, TAF1 seemed to be an important contributor to SRC1A downregulation, as inactivation of TAF1 binding and AT activity prevented further inhibition of SRC1A transcription. Second, functional TAF1 AT or binding activity was not required to negate YES transcription in the presence of HDIs. Third, LYN transcription was strangely stimulated by TSA in both BHK-21 and *tsBN462* cells. This was the first such observation of SFK member gene activation following HDI exposure. In SW480 cells, LYN promoter activity is

decreased by butyrate and TSA; therefore HDI mediated LYN promoter activation may be explained by the absence or presence of a particular factor in BHK-21 cells, compared to SW480 cells. As a whole these data suggest that the AT activity of TAF1 is not universally responsible for suppressing transcription of the SFK members in the presence of HDIs, and therefore likely does not acetylate a non-histone protein at the SRC1A promoter to mediate transcriptional repression. However, it cannot be ruled out that another HAT or AT may still acetylate a non-histone substrate associated with transcription, to suppress the promoter activity of any or all of the SFKs examined.

#### **5.3.3.2. Other Potential Mechanisms of Histone Deacetylase Inhibitor Mediated SRC Repression**

Another possible mechanism of HDI action has focused on the role of HDACs in HDI mediated gene repression. A collection of recent observations have identified a role for HDACs in the activation of particular genes. The yeast HDACs Hos2 and Rpd3 have both been implicated in selective gene activation (Wang *et al.*, 2002 and De Nadal *et al.*, 2004). Trans-activation of STAT5 has also been reported to require HDAC activity for the recruitment of TBP and RNA polymerase II (Rasche *et al.*, 2003). Furthermore, HDAC activity has been shown to be necessary for a number of interferon stimulated response genes (Nusinzon and Horvath, 2003; Klampfer *et al.*, 2004; Sakamoto *et al.*, 2004 and Nusinzon and Horvath, 2005). Therefore, HDIs may suppress the expression levels of genes that require HDAC activity for basal transcription by purely inhibiting the activity of HDACs associated with the gene promoter. Such an observation has been reported for the STAT5 and interferon responsive genes (Rasche *et al.*, 2003 and Nusinzon *et al.*, 2005). Consequently, localization of the Class I and Class II HDACs and their ability to activate the SRC1A promoter has been studied (Ellis and Bonham unpublished data). However, none of the Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) or Class II HDACs (HDAC5, HDAC6, HDAC7 and HDAC10) examined have been found to reside at the SRC1A or SRC1 $\alpha$  promoters either prior to or following HDI exposure, although this may be due to poor quality ChIP antibodies. Encouragingly, siRNA mediated inhibition of Class IIb HDAC member expression resulted in a decrease in c-Src gene expression levels; however, this effect was not mediated by suppression of SRC1A or SRC1 $\alpha$  promoter activity. Furthermore, siRNA



knockdown of the Class I HDACs led to an upregulation in c-Src expression. Also, overexpression of the Class I and Class II HDACs either had little effect on SRC1A and SRC1 $\alpha$  transcription, or decreased promoter activity slightly. Therefore, HDAC activity of neither the Class I nor Class II HDACs appears to be a necessary component of SRC1A or SRC1 $\alpha$  basal transcription and hence cannot play a subsequent direct role in HDI transcriptional repression of this gene.

Other HDI molecular mechanisms suggest that HDIs may affect gene expression levels by disrupting HDAC-PP1 complexes associated with gene promoter regions, as in the case of CREB responsive genes (Canettieri *et al.*, 2003 and Brush *et al.*, 2004). PP1 has been shown to form complexes with HDAC1, HDAC6 and HDAC10; HDIs are capable of disturbing these HDAC-PP1 complexes (Brush *et al.*, 2004). Specifically, the dissociation of HDAC-PP1 components from one another has been suggested to permit CREB transcription factor phosphorylation, HAT recruitment, histone acetylation and transcription (Canettieri *et al.*, 2003 and Brush *et al.*, 2004). PP1 dissociation from the HDACs also initiates an interaction between PP1 and Akt, which leads to Akt inactivation (Canettieri *et al.*, 2003 and Chen *et al.*, 2005). In addition to this, butyrate and valproic acid have been shown to decrease the gene expression levels of two kinases, Akt1 and Akt2 (Chen *et al.*, 2006). Importantly, Akt interacts with and phosphorylates the co-activator p300 to stimulate its HAT activity (Chen *et al.*, 2004 and Huang and Chen, 2005). Therefore, HDIs have the capability to affect acetylation as well as phosphorylation levels in the cell by either disturbing PP1-HDAC complexes or by inhibiting Akt expression. Cumulatively, these data suggest that HDIs have the potential to selectively regulate gene expression levels and signaling pathways by altering the phosphorylation patterns of yet unidentified proteins, including additional transcription factors, signaling molecules, histones, transcriptional co-activators, and components of the general transcription machinery (Alao *et al.*, 2006).

In the course of studying the molecular mechanism behind HDI mediated repression of c-Src gene expression levels, the presence of actively transcribing Pol II along the SRC gene, from Exon1 $\alpha$  and Exon1A to the 3' UTR, was investigated (Ellis and Bonham, unpublished data). A previous report has documented that the loss of Pol II at the transcription start site of the STAT5 promoter is responsible for

downregulation of STAT5 gene expression in the presence of HDIs (Rascole *et al.*, 2003). Under normal cellular conditions, Pol II was readily detected at the SRC1 $\alpha$  and SRC1A promoters, coding region, intronic region and the 3' UTR (Ellis and Bonham, unpublished data). Furthermore, Pol II was detected at Exon 1 $\alpha$  and Exon 1A following exposure to TSA, however, an intermittent drop in Pol II occupancy was observed within the coding region and the 3' UTR after as little as 15 minutes of TSA exposure, and after 1 h of TSA treatment Pol II was barely detectable at the SRC 3' UTR.

In addition to tracking Pol II down the SRC gene, the phosphorylation status of the Pol II unstructured CTD was monitored (Ellis and Bonham, unpublished data). Phosphorylation of the Pol II CTD has been proposed to delineate two important events. The hypophosphorylated form of the Pol II CTD is integrated into the PIC. Upon proper assembly of the PIC, TFIIF phosphorylates Ser 5 residues on the CTD, to facilitate promoter clearance and trigger early elongation (Svejstrup, 2004 and Meinhart *et al.*, 2005). Phosphorylated Ser 5 also acts as a platform for the recruitment of mRNA capping enzymes (Phatnani and Greenleaf, 2006). In addition, the active phosphorylated form of P-TEFb phosphorylates Ser 2 on the Pol II CTD to overcome an early pause in elongation after the nascent mRNA chain has been properly capped (Peterlin and Price, 2006). Therefore, Ser 5 phosphorylation serves as a rough marker for promoter clearance and Ser 2 phosphorylation of the CTD behaves as a marker for productive elongation (Shilatifard, 2004 and Sims *et al.*, 2004). When the phosphorylation status of Pol II CTD along the SRC gene was examined, Ser 5 phosphorylation of the CTD was detected both prior to and following TSA treatment; however, in the presence of TSA a drop in Ser 5 phosphorylation was noted in the 3' UTR, which corresponded with the decrease in Pol II occupancy. Furthermore, similar observations were observed for Ser 2 CTD phosphorylation along the SRC gene, although Ser 2 phosphorylation levels were comparatively much lower than Ser 5 phosphorylation levels at the SRC1 $\alpha$  and SRC1A promoters. As a whole, these data suggest that while SRC1 $\alpha$  and SRC1A transcriptional initiation occurs in the presence of HDIs, productive elongation may not, as a gradual loss of Pol II at the 3' UTR of SRC indicates that formation of incomplete transcripts may be the reason for c-Src gene repression

#### **5.3.3.3. The Working Model of Histone Deacetylase Inhibitor Mediated Downregulation of c-Src Gene Expression**

It is known that c-Src and SFK member gene expression is inhibited by HDIs and that HDIs directly suppress the promoter activity of SFK members in transient transfections assays performed with human tumor cells. However, the mechanism of HDI mediated SRC gene repression is still under investigation. Over the past five years, a number of possible mechanisms have been discounted, including the involvement of *cis*-acting DNA promoter sequences and *trans*-acting DNA binding factors with either of the SRC proximal promoters. In addition, HDI mediated changes in the histone acetylation levels of the SRC promoters do not correspond to the decrease observed in c-Src gene expression levels. Cumulatively, the data collected to date suggest that an event downstream of transcription factor and PIC assembly at the SRC1 $\alpha$  and SRC1A promoters may be targeted by HDIs. A model of HDI control over c-Src expression levels has endured after intensive study; a representation of this mechanism is illustrated for the SRC1A promoter (Figure 5.1.).

This model stipulates that TAF1 and components of the PIC associate via the Inr element of the SRC1A promoter during normal basal transcription and that HDAC-PP1 complexes are present within close proximity to the SRC gene. HDAC-PP1 complexes may maintain an unknown substrate (X) at the SRC1A promoter in a hypophosphorylated and/or hypoacetylated state during formation of the PIC complex. In addition, PP1 has been documented to dephosphorylate residues Ser 5 and Ser 2 of the Pol II CTD, as well as the elongation factor P-TEFb (Washington *et al.*, 2002 and Ammosova *et al.*, 2005). Therefore, HDAC-PP1 complexes may preserve the dephosphorylated form of the Pol II CTD until the PIC has been correctly assembled and loaded with the machinery necessary to properly synthesize and co-transcriptionally process the nascent mRNA transcript. In addition, HDAC-PP1 may inhibit kinase activation of P-TEFb, which phosphorylates Ser 2 residues on the CTD, by preventing the phosphorylation of this factor until the Pol II complex is adequately equipped for productive elongation. Following proper formation of the PIC, TFIIH triggers DNA melting and phosphorylates Ser 5 residues of the CTD to generate an early elongation complex. Subsequently, phosphorylated Ser 5 recruits the mRNA capping enzymes

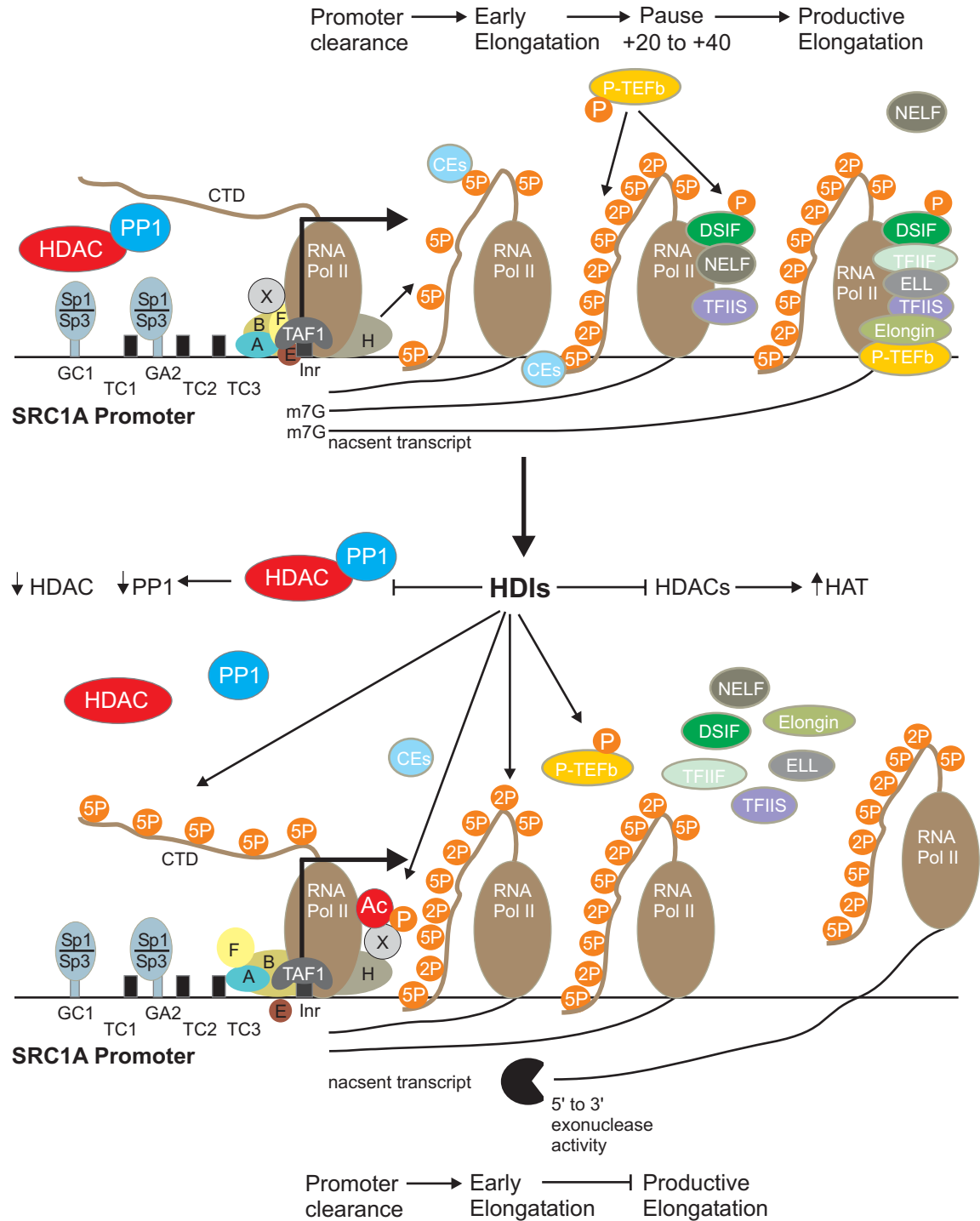


Figure 5.1. The model of histone deacetylase inhibitor mediated SRC gene repression. HDIs alter the phosphorylation and acetylation pattern of non-histone substrates within close proximity to the SRC1A promoter to suppress productive elongation of the SRC gene.

(CEs). Furthermore, NELF, DSIF and TFIIS associate with Pol II to facilitate an early elongation pause which allows capping of the nascent mRNA transcript to proceed. After a 5' 7-methyl-guanosine cap has been added to the pre-mRNA, activated P-TEFb phosphorylates both Ser 2 amino acids on the CTD and DSIF to promote NELF dissociation and re-stimulate Pol II elongation. Productive elongation ensues after further recruitment of elongation factors such as TFIIF, ELL and Elongin.

Here it is proposed that HDIs alter c-Src expression levels by inhibiting HDAC activity and promoting the dissociation of HDAC-PP1 complexes from the SRC1A promoter. In this manner, HDIs may shift the overall equilibrium toward acetylation and facilitate HAT acetylation of an unknown non-histone protein (X) which renders a repressive effect on such events as SRC promoter clearance, nascent mRNA capping or elongation. Although, if acetylation of a non-histone protein contributes to the downregulation of c-Src expression levels, it is unlikely that TAF1 would be the HAT responsible for this acetylation event. In addition to enhancing acetylation levels upon PP1 dissociation from the SRC1A promoter, HDIs may also permit phosphorylation of an unidentified protein substrate (X) which negatively regulates transcript synthesis. Furthermore, loss of PP1 may indirectly allow TFIIH to prematurely phosphorylate Ser 5 residues of the Pol II CTD, before all of the components of the PIC have been correctly assembled. Early phosphorylation of P-TEFb may also stimulate impulsive phosphorylation of Ser 2 on the CTD and ultimately abrogate the early elongation pause, preventing mRNA capping from occurring and opening up the pre-mRNA to exonuclease attack. Alternatively, the processivity of Pol II may be decreased if various elongation factors do not have time to associate with the early elongation complex, motivating Pol II dissociation from the gene. Given that HDI disruption of HDAC-PP1 complexes has previously been reported to stimulate CREB transcription factor phosphorylation, it is plausible that dissociation of HDAC-PP1 components from the SRC1 $\alpha$  and SRC1A promoters may enhance the phosphorylation levels of proteins associated with SRC transcription (Canettieri *et al.*, 2003 and Brush *et al.*, 2004). Taken together, the current evidence suggests that HDIs repress c-Src gene expression by altering the acetylation and/or phosphorylation status of an unidentified non-histone substrate(s) associated with events downstream of transcriptional initiation, such as Pol

II promoter clearance, early elongation, proximal pausing, productive elongation and/or mRNA processing.

#### **5.3.4. The Importance of c-Src Family Kinase Member Gene Repression by Histone Deacetylase Inhibitors**

While the work presented in this thesis primarily focuses on the ability of HDIs to regulate gene expression irrespective of histone hyperacetylation, it is important to comment on the significance of HDI downregulation of SFK gene expression levels. Members of the c-Src family of kinases have highly conserved amino acid sequences and have been suggested to exhibit compensatory effects for one another. SFKs have been extensively implicated in a number of cellular signaling pathways associated with proliferation, differentiation, adhesion, migration, invasion, survival angiogenesis and immune development (Thomas and Brugge, 1997; Biscardi *et al.*, 1999; Frame, 2002 and Yeatman, 2004). Therefore, it is not surprising that overexpression and/or activation of a number of these SFKs have been associated with tumor cell transformation, progression and metastasis (Summy and Gallick, 2003). In particular, high c-Src gene expression levels and/or kinase activity have been reported in colon carcinoma cells, and c-Src kinase activity has been suggested to increase as colon cancer cells progress from a pre-malignant state to primary and metastatic phenotypes (Rosen *et al.*, 1986; Bolen *et al.*, 1987; Cartwright *et al.*, 1989; Cartwright *et al.*, 1990; Talamonti *et al.*, 1993; Cartwright *et al.*, 1994; Han *et al.* 1996 and Mao *et al.*, 1997). Furthermore, an enhancement in c-Src kinase activity has been proposed to be prognostic for poor clinical outcome in patients with colorectal carcinoma (Aligayer *et al.*, 2002). The elevation in c-Src levels may be caused by protein deregulation, a rare Y531 mutation at the C-terminal end of the c-Src protein, or an upregulation in SRC promoter transcription (Irby *et al.*, 1999, Dehm *et al.*, 2001).

Given that c-Src kinase levels are noticeably elevated in human colon cancer cells, studies have been performed to determine if c-Src is an important anti-tumor target. Indeed, antisense experiments have shown that suppression of c-Src expression decreases tumor growth and downregulates levels of the angiogenic factor vascular endothelial growth factor (VEGF) (Staley *et al.*, 1997 and Ellis *et al.*, 1998).

Furthermore, various inhibitors of c-Src kinase activity have been shown to reduce growth, survival, adhesion, migration, invasion and metastasis of tumor cells and a number of these drugs are entering clinical trials (Alvarez *et al.*, 2006 and Summy and Gallick, 2006). Therefore, it was particularly exciting to observe that HDIs effectively downregulated the expression levels of c-Src as well as the SFKs in HCCLs, a finding that further suggests HDIs may be relevant therapeutic agents for the treatment of colon tumors expressing high levels of SFK members (Kostyniuk *et al.*, 2003 and Hirsch *et al.*, 2006). In addition, butyrate is a well established chemo-preventive colon cancer agent that exists at high concentrations in the colon as a result of eating a high fiber diet (Roediger, 1980). Currently it is unclear whether inhibition of the SFK members contributes to the chemo-preventive nature of butyrate, although it would be interesting to decipher if the downregulation in SFK gene expression levels contributes directly to the chemo-preventive and anti-tumor effects observed upon HDI exposure to malignant cells.

#### **5.4. Histone Deacetylase Inhibitor Post-transcriptional Regulation of p21WAF1**

An alternative and previously unreported form of HDI mediated gene regulation was serendipitously discovered as experiments were carried out to investigate the effects of HDIs on c-Src expression levels. Interestingly, p21WAF1, the model gene used as the definitive example to explain the mechanism of HDIs, was the target. As described earlier, the current literature indicates that HDIs directly facilitate p21WAF1 gene activation through Sp family binding sites located within the proximal region of the p21WAF1 promoter. Indeed, it was noted in HepG2 cells that HDIs dramatically induced p21WAF1 mRNA and protein levels, however, new protein synthesis was required in order for this to occur. Furthermore, HDIs could not stimulate p21WAF1 promoter activation in HepG2 cells to anywhere near the extent observed in HT29 cells. Specifically, a very minor butyrate mediated increase (< 2-fold) in p21WAF1 activity was observed, although the more specific HDI, TSA, did not activate the promoter at all in HepG2 cells. Such an indirect effect on p21WAF1 gene expression levels had not been previously reported to be elicited by HDIs. At first, it was hypothesized that HDIs may be incapable of further trans-activating the p21WAF1 promoter in the HepG2 cell

line, given that these cells already display high p21WAF1 promoter activity and constitutively high p21WAF1 expression levels. However, p21WAF1 promoter activity was sufficiently stimulated in HepG2 cells by c-jun, a known super-activator of p21WAF1 transcription, to suggest that the p21WAF1 promoter was still theoretically open to HDI mediated gene activation. Further investigation into the indirect effect that HDIs exert on p21WAF1 expression led to the discovery that HDIs regulate p21WAF1 gene expression at the post-transcriptional level. Interestingly, butyrate and TSA enhanced p21WAF1 mRNA stability 3.1 and 5.4-fold respectively, in HepG2 cells. This was the first reported example of HDI post-transcriptional gene regulation, however, HDI mediated p21WAF1 mRNA stabilization has only been observed in HepG2 cells (Hirsch and Bonham, 2004). Since this time, HDIs have also been shown to reduce the mRNA stability of DMT3B and DMT1, indicating that regulation of mRNA stability may be a more universal action of HDIs than previously anticipated (Xiong *et al.*, 2005 and Januchowski *et al.*, 2007).

#### **5.4.1. The Mechanism of Histone Deacetylase Inhibitor Mediated p21WAF1 mRNA Stabilization**

mRNA *cis*-acting stability elements and the *trans*-acting RNA binding proteins that interact with these sequence determinants have been extensively implicated as powerful regulators of mRNA stability. Stability determinants have been elucidated in the 5' UTR, coding region and 3' UTR of various mRNA species. The p21WAF1 mRNA contains three *cis*-acting destabilizing ARE elements and a HuD binding element within the first 300 nucleotides of its 3' UTR that have been reported to control mRNA decay (el-Deiry *et al.*, 1993 and Joseph *et al.*, 1998). AUF1 and the ELAV family members HuR and HuD are three known *trans*-acting factors that bind to these *cis*- nucleotide sequences of the 3' UTR to regulate p21WAF1 mRNA stability (Wang *et al.*, 2000; Giles *et al.*, 2003 and Lal *et al.*, 2004). The interaction of AUF1 with the p21WAF1 3' UTR has been shown to decrease mRNA stability, however, HuR interaction with the proximal region of the 3' UTR causes an increase in the mRNA stability of p21WAF1 following UVC or prostaglandin A<sub>2</sub> exposure (Wang *et al.*, 2000; Lal *et al.*, 2004 and Yang *et al.*, 2004). In our hands, the p21WAF1 3' UTR was also shown to possess strong destabilizing potential when it was inserted into a -210WAF1-



CAT or pCAT3 promoter vector downstream of the vector CAT coding region, when used in a HepG2 transient transfection system. Furthermore, the presence of the p21WAF1 3' UTR downstream of the -210WAF1-CAT increased CAT expression levels 6.9 and 11.7-fold in transfected HepG2 cells upon TSA and butyrate exposure, respectively. Taken together, these observations verified that p21WAF1 mRNA stability could be effectively monitored at the level of CAT activity and suggested that a *cis*-acting element within the p21WAF1 3' UTR may be responsible for mediating basal mRNA stability as well as the effect of HDIs on p21WAF1 mRNA stabilization. Given that HDIs were previously shown to indirectly stimulate mRNA stability of p21WAF1 and that this required new protein synthesis, it was proposed that a newly synthesized *trans*-acting RNA binding protein may interact with a *cis*-acting element in the 3' UTR to facilitate stabilization of the mRNA species.

#### **5.4.1.1. Histone Deacetylase Inhibitors Mediated Stabilization of p21WAF1 mRNA by Two *cis*-acting Sequences in the p21WAF1 3' Untranslated Region**

The search for a HDI responsive *cis*-acting element was initialized by systematic deletion of p21WAF1 3' UTR proximal nucleotides from the -210WAF1-CAT + p21WAF1 3'UTR reporter plasmid. Removal of the HuD element and all of the ARE sites from the p21WAF1 3' UTR had neither a considerable effect on the overall basal mRNA stability of p21WAF1, nor prevented the increase observed in p21WAF1 mRNA stability upon exposure to HDIs. Surprisingly, the reporter plasmid lacking the HuD site and all three AREs displayed only a very minor increase in basal mRNA stability, and an approximate 50% reduction in p21WAF1 mRNA stability in the presence of HDIs. However, when just the 250 proximal nucleotides of the 3' UTR that contain the HuD and ARE elements were inserted into a -210WAF1-CAT + p21WAF1 3'UTR vector, a massive stabilization in basal p21WAF1 mRNA levels was observed and neither TSA nor butyrate were able to boost mRNA stability. Although the most influential *cis*-acting stability elements have been reported to reside within the proximal 300 nucleotides of the p21WAF1 3' UTR, these results suggest that the remaining distal nucleotides of the p21WAF1 3' UTR, between nucleotides 840 and 2079, contain a *cis*-acting sequence stability determinant responsive to HDIs. This was not the first time that sequence downstream from the HuD site and AREs have been suggested to contain

additional instability determinants. Other groups have also shown that exclusion of the proximal portion of the 3' UTR still leads to prompt p21WAF1 mRNA decay (Li *et al.*, 1996 and Giles *et al.*, 2003). In addition to this, EGF has been reported to mediate p21WAF1 mRNA stabilization through multiple *cis*-acting determinants in the distal portion of the 3' UTR (Giles *et al.*, 2003).

Further deletions were made to search the distal region of the p21WAF1 3' UTR for this *cis*-acting element. The exclusion of large segments of the 3' UTR between nucleotides 591-1272 identified that nucleotides 1045-1272 of the p21WAF1 3' UTR harbor an instability determinant and that nucleotides 932-1272 contain a *cis*-acting HDI response element. However, when a p21WAF1 3' UTR fragment (840-1470) containing sequence corresponding to these regions was inserted into the -210WAF1-CAT vector and transiently transfected into HepG2 cells, the inherent instability of the p21WAF1 full length 3' UTR was not recovered and the HDI response was not rescued. These results hint at the presence of an additional *cis*-acting element between nucleotides 1272 and 2079 of the p21WAF1 3' UTR, a region which also mediates p21WAF1 basal mRNA stability and HDI induced mRNA stabilization.

Indeed, further examination of the p21WAF1 3' UTR identified the presence of a second *cis*-acting element within the extreme 3' end of the p21WAF1 3' UTR. Located between nucleotides 2000 and 2079, this element controls both basal and HDI responsive p21WAF1 mRNA stability. However, in accordance with the previous data, a fragment of the p21WAF1 3' UTR (1468-2079) containing the extreme 3' nucleotides was unable to return basal p21WAF1 mRNA to low stability levels, nor enhance p21WAF1 mRNA stability when exposed to HDIs. This suggests that at least two *cis*-acting elements are required to mediate mRNA stabilization. An alignment of the human, rat and mouse p21WAF1 3' UTRs revealed high sequence similarity across all three species, particularly within the region immediately upstream of the poly (A) site hexamer that housed the most distal *cis*-acting element. Accordingly, the most distal element of the p21WAF1 3' UTR was further characterized by methodically mutating the nucleotides between 2001-2079 of the 3' UTR, five consecutive base pairs at a time. Surprisingly, the nine nucleotides immediately upstream of the poly (A) site hexamer 2071-2079 were found to encompass the distal *cis*-acting element, as the consecutive

mutation of these nucleotides resulted in an increase in p21WAF1 basal mRNA stability and a reduction in HDI induced p21WAF1 mRNA stabilization. Given the immediate proximity of the p21WAF1 poly (A) site hexamer sequence, it is also interesting to note that this distal *cis*-acting element did not require the context of the p21WAF1 poly (A) site hexamer directly neighboring it to mediate an effect on mRNA stabilization.

Of further mention is the observation that -210WAF1-CAT + p21WAF1 3'UTR vectors which generated low basal levels p21WAF1 mRNA stability such as 591-2079 often directly corresponded to high HDI mediated stabilization of p21WAF1 mRNA levels. Furthermore, plasmids, such as -210WAF1-CAT + p21WAF1 3'UTR 591-840 and -210WAF1-CAT + p21WAF1 3'UTR 591-2000 had a higher basal stabilization level, but were relatively defective enhancers of p21WAF1 mRNA stability in the presence of HDIs. Therefore, common *cis*-acting elements that mediate both basal p21WAF1 mRNA stability and HDI responsive stabilization may exist and changes in *trans*-factor binding to these *cis*-acting elements may ultimately dictate the mRNA stability of p21WAF1.

In traversing the entire p21WAF1 3' UTR, both 5' and 3' deletions were made to the 3' UTR, and two distinct fragments were identified as important *cis*-acting regulators of mRNA stability in the absence and presence of HDIs. One *cis*-acting fragment was discovered between nucleotides 932-1272 and the other between nucleotides 2071-2079 of the p21WAF1 3' UTR. Given the previous hypothesis that HDIs regulate p21WAF1 mRNA stability via *cis*-acting elements and the *trans*-factors that bind them, RNA binding assays were completed in order to elucidate changes in protein binding after HDI treatment. However, an alteration in *trans*-factor binding was not observed across the length of the entire p21WAF1 3' UTR when RNA fragments of the 3' UTR corresponding to nucleotides 586-840, 840-1481, and 467-2079 were examined. These data, in correlation with earlier transfection data, suggest once again that the *cis*-acting elements found between nucleotides 932-1272 and 2071-2079 are both required to mediate p21WAF1 mRNA stability in the absence and presence of HDIs.

#### 5.4.2. The Model of Histone Deacetylase Inhibitor Regulation of p21WAF1 mRNA Stability

Histone deacetylase inhibitors have been shown to require protein neo-synthesis to mediate HDI p21WAF1 mRNA stabilization in HepG2 cells. Two novel *cis*-acting elements within the p21WAF1 3' UTR have also been identified as critical determinants of basal and HDI mediated mRNA stability, however, the presence of neither of these elements alone is sufficient to control mRNA decay; this likely requires the entire context of p21WAF1 3' UTR nucleotides 932-2079. Therefore, the secondary and/or tertiary structure of the p21WAF1 mRNA may also be an important factor in p21WAF1 mRNA stabilization. Interestingly, the predicted secondary structure of the p21WAF1 mRNA as determined by MFOLD, a program that creates thermo-dynamically favorable mRNA secondary structures, suggests that the two newly identified *cis*-acting elements 932-1272 and 2071-2079 fold within close proximity to one another (Zuker *et al.*, 1999) (<http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html>) (Figure 5.2.). This model proposes that inhibition of HDAC activity leads to an upregulation in the expression of an unidentified protein that binds the p21WAF1 mRNA within the vicinity of both *cis*-acting elements. In order for these *cis*-elements to facilitate an interaction, either directly or indirectly, with this unknown protein, nucleotides 932 to 2079 of the 3' UTR must be present to ensure correct folding of the mRNA. However, proper folding of the mRNA ensures *trans*-protein binding to p21WAF1 mRNA, which may subsequently enhance p21WAF1 mRNA stability by preventing destabilizing proteins or alternatively microRNAs (miRNAs) from accessing the 3' UTR in the presence of HDIs. Although, in the absence of HDIs, destabilizing proteins and/or miRNAs are able to access the p21WAF1 3' UTR and lower p21WAF1 basal mRNA stability levels are maintained.

miRNAs are some of the most recently recognized contributors to post-transcriptional mRNA regulation. These RNAs are approximately 21-26 nucleotides in length and have been reported to stimulate mRNA decay via deadenylation, endonucleolytic cleavage or repression in protein synthesis following the formation of double stranded RNA duplexes by antisense binding with precise 3' UTR sequences of mRNAs (Gupta and Brewer, 2006; Roush and Slack, 2006; Valencia-Sanchez *et al.*,

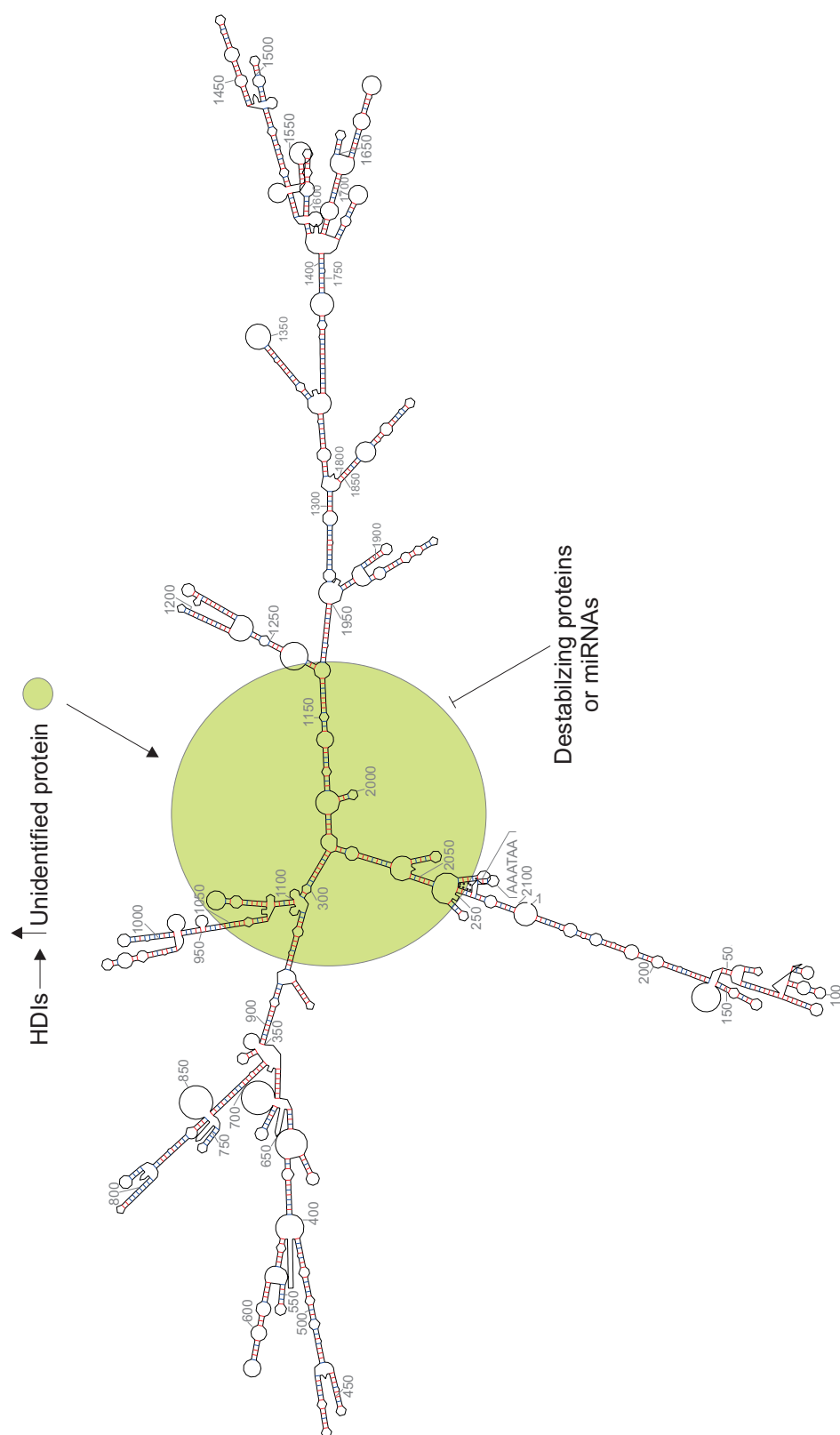


Figure 5.2. The model of histone deacetylase inhibitor induced p21WAF1 mRNA stabilization. HDIs stimulate the expression of an unknown protein, which binds to the p21WAF1 mRNA secondary structure. The association of this protein with the 3' UTR prevents p21WAF1 mRNA decay. Two 3' UTR *cis*-acting determinants between nucleotides 932-1272 and 2071-2079 have been implicated in HDI mediated p21WAF1 mRNA stabilization. The predicted p21WAF1 mRNA secondary structure was determined using MFOLD (Zuker *et al.*, 1999) (<http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html>)

2006 and Wu *et al.*, 2006). The p21WAF1 3' UTR is a potential target of fourteen different miRNAs, as determined by the Sanger miRBase. Interestingly, ten of these miRNA target sequences fall between nucleotides 932 and 2079 of the p21WAF1 3' UTR and four of these (miR-525 and miR 526a: target nucleotides 918-934, miR-423: 1048-1062, miR-409-5p: 1101-1122, miR-505: 1269-1290) reside in close proximity to both *cis*-acting stability determinants when the p21WAF1 mRNA is folded into its secondary structure. In addition, nucleotides 1508 to 1529 of the p21WAF1 3' UTR are proposed to interact with the miRNA miR-22, the expression of which has been noted in the liver. This may provide a plausible explanation for why HDI induced p21WAF1 mRNA stabilization has only been observed in the hepatocellular carcinoma HepG2 cell line (Rodriguez *et al.*, 2004). Furthermore, HDIs have been shown to downregulate the expression levels of miR-22 and 21 other miRNAs, and to upregulate the expression of 5 additional miRNAs (Scott *et al.*, 2006). Therefore, HDIs may post-transcriptionally regulate p21WAF1 mRNA stability at multiple levels, however, the presence of two *cis*-acting elements between nucleotides 932-1272 and 2071-2079, as well as the secondary structure of the 3' UTR, appear to be central to mediating p21WAF1 mRNA stabilization in HepG2 cells.

### 5.5. Scope and Significance

HDIs are complex chemo-therapeutic compounds that selectively regulate the expression levels of genes, such as p21WAF1, to ultimately prevent tumor cell growth and encourage cellular differentiation and/or apoptosis. However, the molecular mechanism of HDI action is not as simplistic as previously proposed. In fact, HDIs are capable of controlling multiple facets of gene expression, aside from mediating histone hyperacetylation and activating gene transcription. Given that HDIs have quickly become realistic therapeutic agents, it is increasingly apparent that the actions of HDIs need to be elucidated. Here, two additional modes of HDI gene regulation were discussed. HDIs were shown to repress the gene expression levels of c-Src and the SFK members in HCCLs, and were documented to enhance p21WAF1 mRNA expression at the post-transcriptional level in HepG2 cells.

## 6. CONCLUSIONS AND FUTURE STUDIES

HDIs are exciting anti-tumor drugs that have received a great deal of attention in clinical trials, due to their ability to specifically stimulate cell cycle arrest, differentiation and/or apoptosis of tumor cells, without affecting normal cells (Marks *et al.*, 2001; Burgess *et al.*, 2004 and Carey and La Thangue, 2006). SAHA is a HDI from the hydroxamic acid family that has rapidly gone through clinical trials and has recently become the first HDI accepted as a therapeutic agent for the treatment of a human malignancy, CTCL (Garber, 2007 and Marks and Breslow, 2007). The study of HDIs is gaining momentum in the clinic and it is almost ensured that additional HDIs will also be accepted for clinical use, either as monotherapies or in combination with other chemotherapies. Therefore, it is imperative that the molecular actions of HDIs are clearly understood. For decades HDIs have been thought to mediate their anti-cancer effects through the regulation of gene expression patterns. HDIs have been proposed to control gene expression primarily by inhibiting the activity of HDACs and thereby shifting the overall balance between HATs and HDACs in the cell to promote histone hyperacetylation. Enhanced histone acetylation of lysine residues negates the positive charge of the amino terminal histone tails, reducing the electrostatic interaction between these histone tails and the negatively charged DNA backbone. Accordingly, this hyperacetylated histone state presents an open and accessible DNA backbone for the transcriptional machinery to interact with, and thus encourages the activation of gene transcription. However, HDIs have been reported to alter the expression levels of only a small percentage of genes and are able to both activate and repress genes (Van Lint *et al.*, 1996; Mariadason *et al.*, 2000 and Peart *et al.*, 2005). In addition, a number of reports have surfaced documenting a requirement for PKC isoforms  $\epsilon$  and  $\delta$  in HDI mediated gene regulation (Han *et al.*, 2001 and Kim *et al.*, 2003, Eun *et al.*, 2007 and

Kim *et al.*, 2007). Therefore, it has become increasingly apparent that HDIs regulate gene expression by additional molecular mechanisms, irrespective of histone hyperacetylation.

In this thesis, the HDIs butyrate and TSA were shown to suppress the gene expression levels of the c-Src proto-oncogene in a dose and time dependent manner in a variety of human tumor cell lines including colon, breast and hepatocellular carcinoma cells. Specifically, independent of *de novo* protein synthesis, HDIs directly inhibited transcription from both highly disparate SRC promoters, SRC1 $\alpha$  and SRC1A, in transient transfection assays. In addition to inhibiting c-Src gene expression, HDIs also downregulated the expression levels of the SFK members Yes, Fyn, Lyn and Lck in HCCLs. Analogous to what was found with c-Src, new protein synthesis was not required to facilitate HDI repression of Lyn and Lck mRNA expression. HDIs also impaired the activity of the LCK type I, LYN and YES promoters in transient transfections.

A direct comparison of the SFK member promoters revealed that all the promoters were TATA-less and contained multiple transcription start sites (Matsuzawa *et al.*, 1991; Uchiumi *et al.*, 1992; Leung *et al.*, 1993 and Bonham and Fujita, 1997). As Dr. Scott Dehm reported that both the SRC1A and SRC1 $\alpha$  promoters were driven by an Inr element that required TAF1 to facilitate basal transcription, and further suggested that TAF1 may be a necessary factor for HDI mediated repression of the SRC1A promoter, TAF1 dependency of the other SFKs was also examined (Dehm *et al.*, 2004). Fittingly, the YES and LYN promoters were identified to be TAF1 dependent in nature. However, neither HDI mediated downregulation of YES nor LYN required TAF1. Results obtained from transient co-transfections with TAF1 and TAF1 mutant AT activity expression vectors, also strongly suggested that functional AT activity of TAF1 was required to stimulate SRC1A, YES and LYN transcription. However to conclusively show that TAF1 AT activity is required for basal SFK member transcription, *in vitro* binding assays must be performed to ensure that TAF1 AT mutants still retain their ability to interact with the SRC1A Inr element. These experiments are currently being carried by Dr. Edith Wang at the University of Washington. The mechanism of HDI mediated repression of the SFK members is still



unclear, however, work performed by Dr. Scott Dehm and Danielle Ellis cumulatively suggests that HDIs decrease c-Src gene expression levels by preventing complete synthesis of SRC transcripts. While the mechanism of SRC gene repression is currently being studied, it will be of future interest to establish if HDIs mechanistically regulate SFK members in a similar manner, and to investigate if HDIs commonly downregulate the expression of other genes through the same mechanism.

It is known that HDIs exhibit powerful anti-cancer effects and that butyrate is a well described naturally produced chemo-preventive agent in the colon. Furthermore, overexpression and/or kinase activation of c-Src and the SFK members has been frequently described in colorectal carcinoma, and downregulation of c-Src expression has been shown to decrease tumor cell growth. This suggests that HDI downregulation of SFK members may be of clinical significance; however, it is currently uncertain whether HDI mediated inhibition of c-Src and/or SFK gene expression levels directly accounts for the anti-tumor or chemo-preventive nature of HDIs. To establish if a potential link exists between SFK gene repression and the anti-tumor effects of HDIs, such as butyrate in colon cancer, stable cell lines could be created in HT29 cells containing the SRC gene fused to a promoter known to be transactivated by HDIs, such as p21WAF1. A direct comparison between untransfected and stably transfected HT29 cells following HDI treatment would offer a perspective on whether the prevention of HDI mediated c-Src inhibition alters the ability of HDIs to induce cell cycle arrest, differentiation and/or apoptosis in a HCCL.

In addition to presenting the finding that HDIs suppress SFK member expression levels in HCCLs in a non-conventional manner, this thesis also reports that HDIs upregulate p21WAF1 expression at the post-transcriptional level in HepG2 cells. HDIs are well known activators of p21WAF1 gene transcription, and p21WAF1 expression has been documented to be a prerequisite for HDI stimulated cell cycle arrest (Nakano *et al.*, 1997; Sowa *et al.*, 1997; Archer *et al.*, 1998 and Huang *et al.*, 2000). However, while HDIs were shown to be incapable of trans-activating the p21WAF1 promoter in HepG2 cells, they remained proficient at upregulating p21WAF1 mRNA and protein levels. In a unique manner, HDIs indirectly enhanced p21WAF1 expression levels by stabilizing p21WAF1 mRNA levels in HepG2 cells.

p21WAF1 mRNA stability has been reported to be regulated by *cis*-acting stability determinants in the 3' UTR and the *trans*-acting factors that bind them. In particular, a HuD site and three AREs are important *cis*-acting elements within the proximal region of the p21WAF1 3' UTR that have been extensively documented to interact with *trans*-associated factors and elicit an effect on the mRNA stabilization of p21WAF1. As presented in this thesis, two novel *cis*-acting elements in the 3' UTR of the p21WAF1 mRNA were also identified as critical regulators of basal and HDI induced p21WAF1 mRNA stability. These *cis*-acting fragments of the p21WAF1 mRNA were initially localized to nucleotides 932-1272 and 2000-2079 of the 3' UTR. Further characterization narrowed down a region between nucleotides 2071 to 2079 of the p21WAF1 3' UTR as the distal *cis*-acting element. Although both of these *cis*-acting elements, 932-1272 and 2071-2079, were independent critical mediators of p21WAF1 stability, neither of these elements alone was sufficient to induce p21WAF1 mRNA stabilization in the presence of HDIs. Furthermore, a change in *trans*-binding factors was not detected at individual *cis*-acting elements before and after HDI exposure, further suggesting that these *cis*-acting elements are not mutually exclusive. Investigation of the predicted p21WAF1 mRNA secondary structure identified that both *cis*-acting elements reside in close proximity. Therefore, the *cis*-acting elements between 932-1272 and 2071-2079, as well as proper folding of the p21WAF1 mRNA, may collaboratively mediate p21WAF1 mRNA stabilization in the absence and presence of HDIs in HepG2 cells.

To investigate the validity of this hypothesis, RNA binding assays should be performed with fragments of the p21WAF1 mRNA containing the entire 932-2079 stretch of 3' UTR ribonucleotides. This length of the 3' UTR sequence should allow proper folding of the RNA and provide a theoretically receptive template to observe changes in *trans*-factor binding in the absence and presence of HDIs. However, the *in vitro* synthesis of a RNA template greater than 500 nucleotides is quite difficult to generate. Also, it would be of benefit to identify the precise nucleotides of the 932-1272 *cis*-acting element responsible for p21WAF1 mRNA stability in HepG2 cells. Sequential mutation of the bases between 932 and 1272 of the 3' UTR could serve as a method for elucidation, just as the 2071-2079 *cis*-acting sequence was detected. In

addition, given that the p21WAF1 3' UTR possesses a large number of potential miRNA target sequences it would also be interesting to determine whether mutation of any of these sequences affects p21WAF1 mRNA stability or prevents HDI induced p21WAF1 mRNA stabilization. Furthermore, as HDIs have only been observed to stabilize p21WAF1 mRNA levels in HepG2 cells, it is important to determine whether this effect is cell specific or more universal. To start, liver cell lines and other cell lines which express relatively high levels of p21WAF1 could be investigated.

The field of HDI research has undergone a great deal of evolution over the past ten years and will continue to develop with an improved perspective on the mechanisms of HDI action. Dogmatically, HDIs have been thought to mediate their anti-tumor effects by inducing changes in the pattern of gene expression by causing histone hyperacetylation and transcriptional activation. However, the observations presented in this thesis highlight that HDIs are more complex chemo-therapeutic compounds than previously anticipated and that they selectively control multiple facets of gene regulation to ultimately prevent tumor cell growth, and encourage differentiation and apoptosis.

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